CHAPTER IV

RESULTS AND DISCUSSION

In Vitro Studies

1. Determination of Suitable Internal Standard (IS), HPLC Condition, and Procedure to Measure Serum Methotrexate Concentration.

The conditions and procedures to determine serum methotrexate concentration were applied from the methods of Canfell, Chen, Cohen, Collier, Howell, Lawson, and Watson as follow (Appendix D).

1.1 Using the Conditions of which Applied from Watson, Cohen, and Chen (Watson et al., 1978 ; Cohen et al., 1980; Chen et al., 1981).

1.1.1 Condition I.

column : ODS

detector : ultraviolet spectrophotometer, at 305 nm

flow rate : 1 ml/min

solvent : 0.025 M phosphate buffer pH 6.25/MeOH = 2/1

tested IS : sulfadiazine, phenacetin, trimethoprim, 4-aminoantipyrine, or methylparaben

result : Result of determination of suitable internal standard was shown in Table 1, and Figure 5.

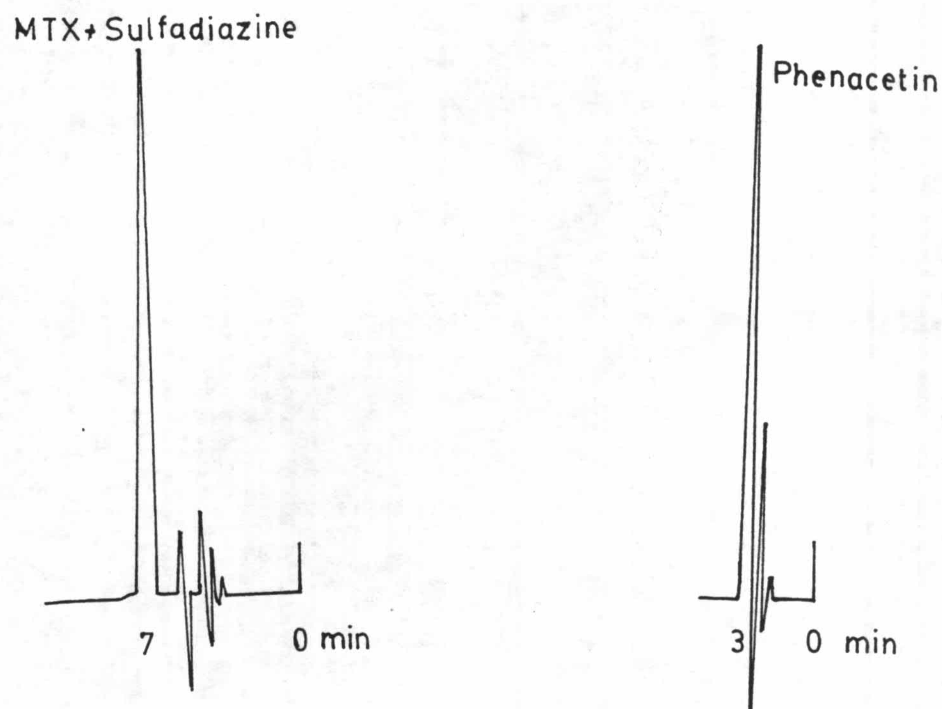

Figure 5. Chromatogram of sulfadiazine, phenacetin as internal standard in condition of which applied from Watson, Cohen, and Chen (condition I).

Table 1. Results of determination of suitable internal standard in condition of which applied from Watson, Cohen and Chen (condition I).

Internal standards	Retention times (minutes)	Notice
sulfadizine	7	Retention time of MTX was 7 mins.
phenacetin	3	
4-aminoantipyrine	30	
methylparaben	27	
trimethoprim	-	

Retention time (RT) of sulfadiazine (7 mins) was equal to that of methotrexate, both peaks were overlapped. Retention time of phenacetin (3 mins) was short, its peak might be obscured by serum peak. Retention time of 4-aminoantipyrine and methylparaben were too long (30, and 27 mins). In this condition trimethoprim could not be eluted. All 5 reagents were not suitable internal standards.

1.1.2 <u>Condition II.</u>

column : radial μ Bondapak C18

detector : ultraviolet spectrophotometer, at 303 nm

flow rate : 1.8 ml/min

solvent : 0.5 M phosphate buffer pH 6.6/MeOH = 75/25

tested IS : sodium aminosalicylate, p-aminosalicylic acid, p-aminoacetophenone (PAAP), or thiopentone sodium

result : Result of determination of suitable internal standard was shown in Table 2, and Figure 6. Retention time of sodium aminosalicylate and p-aminosalicylic acid were short (3 mins). Their bands might be obscured by serum band. In this condition thiopentone sodium could not be eluted. Of all tested internal standard, p-aminoacetophenone could be internal standard but not the best one. Its retention time was 15 minutes and its band was not interfered with serum band. p-Aminoacetophenone has a resolved peak.

Table 2. Results of determination of suitable internal standard in condition of which applied from Watson, Cohen and Chen (condition II).

Internal standards	Retention times (minutes)	Notice
sodium aminosalicylate	3	RT of MTX was 7
p-aminosalicylic acid	3	minutes.
PAAP	15	
thiopentone sodium	-	

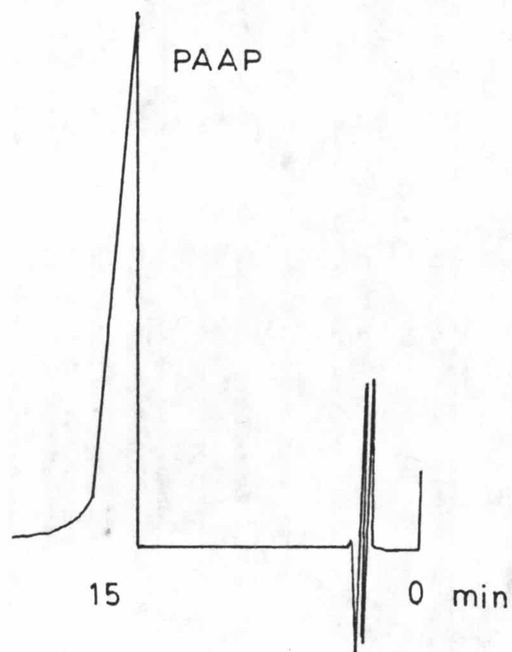

Figure 6. Chromatogram of p-aminoacetophenone (PAAP) as internal standard in condition of which applied from Watson, Cohen, and Chen (condition II).

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1.1.3 Condition III.

column : radial μ Bondapak C18

detector : ultraviolet spectrophotometer, at 303 nm

flow rate : 1 ml/min

solvent : 0.25 M phosphate buffer pH 6.8/MeOH = 77/23

tested IS : p-aminoacetophenone

Extractions and results were shown in Tables 3 - 4, and Figures 7 - 20. Retention times of methotrexate and p-aminoacetophenone were 13 and 17 minutes, respectively. Serum protein was precipitaed using acetonitrile then the clear solution was injected into HPLC system. Methotrexate band could not be detected (Figure 7) because methotrexate was diluted with acetonitrile.

Evaporating the supernatant would increase methotrexate concentration, but serum band was broad and interfered methotrexate band (Figure 8, 9) and peak of methotrexate was short. In evaporation, it took long time about 1 to 1.5 hours till the solution was dried because the supernatant consisted of water phase (serum), and organic phase (acetonitrile or MeOH).

When serum protein was precipitated with precipitating agent, pH of the supernatant might be adjusted to be neutral, after that the supernatant was extracted with organic solvent, next the organic phase was evaporated to dryness to concentrate methotrexate

concentration. From Table 4, extracting serum methotrexate with organic solvent would reduce serum band width (Figures 10 - 17).

When serum was precipitated with trichloroacetic acid, retention time of methotrexate and p-aminoacetophenone were varied if pH of the supernatant was not adjusted to be neutral (Figure 11). But if pH of the supernatant was adjusted with NaOH, next the supernatant was extracted with ethyl acetate/isopropanol (10/1), after that the extracted organic phase was evaporated to dryness and the residue was reconstitueted in solvent, the chromatogram (Figure 10) showed a little serum band width, but serum band interfered methotrexate band. Bands of methotrexate and p-aminoacetophenone were big, and recovery of methotrexate was only 29.7%.

Trichloroacetic acid could precipitate proteins in the serum to be better than perchloric acid ($HClO_4$) [Figure 12]. Serum protein was precipitated with perchloric acid, and pH of the supernatant was adjusted with NaOH, only small amount of methotrexate and p-aminoacetophenone were extracted into chloroform or chloroform/isopropanol layer (Figure 13, 14).

When serum protein was precipitated with perchloric acid, then pH of the supernatant was adjusted with Na_2CO_3, chemically reaction of Na2CO3 with $HClO_4$ produced CO_2 and band of unknown product

which obscured methotrexate band (Figure 15).

When serum protein was precipitated with perchloric acid, then pH of the supernatant was adjusted with excess ammonium sulfate, next the supernatant was extracted with ethyl acetate/isopropanol and the organic layer was evaporated to dryness, after that the residue was reconstituted in phosphate solution. The serum band was broad and interfered methotrexate and p-aminoacetophenone bands (Figure 16). If pH of the supernatant was not adjusted after precipitating serum with perchloric acid, when the the supernatant was extracted with ethyl acetate/ isopropanol, the solution became one phase. The organic phase could not be seperated from the water phase.

Serum protein was precipitated with acetonitrile then the supernatant was extracted with organic solvents. Only small amount of methotrexate could be extracted from ethyl acetate, ethyl acetate /isopropanol or chloroform/isopropanol (Figure 17, 18, 19).

From Table 3, serum protein was precipitated with MeOH, then the supernatant was evaporated to dryness and the residue was reconstituted in phosphate solution, next the phosphate solution was extracted with ethyl acetate/isopropanol (10/1), after that the organic phase was evaporated to dryness and the

Table 3. Extractions and results of procedures to find serum methotrexate level of which applied from Watson, Cohen, and Chen (condition III).

Extractions	Results
MTX 0.1 mcg/serum 1 ml + acetonitrile 1 ml ↓ centrifuged the supernatant was injected into HPLC	MTX peak could not be detected (Figure 7).
MTX 0.1 mcg and PAAP 0.12 mcg/serum 1 ml + acetonitrile 2 ml ↓ centrifuged the supernatant was evaporated to dryness at 60°C ↓ the residue was reconstituted in solvent (water, MeOH, or the solvent for HPLC) 200 μl ↓ the solution was injected into HPLC	Serum band was broad, peak of MTX and PAAP were short. Resolution was 0.7 (Figure 8).
MTX 0.2 mcg and PAAP 0.12 mcg/serum 1 ml + MeOH 2 ml ↓ centrifuged the supernatant was evaporated to dryness at 60°C ↓	Serum band was broad and interfered MTX band (Figure 9).

Table 3 (cont.). Extractions and results of procedures to find serum methotrexate level (condition III).

Extractions	Results
the residue was reconstituted in MeOH 100 µl ↓ the solution was injected into HPLC	
MTX 1 mcg and PAAP 0.12 mcg/serum 1 ml + precipitating agent ↓ centrifuged the supernatant + pH adjusting agent ↓ the supernatant was extracted with organic solvent 2 ml ↓ the organic phase was evaporated to dryness at 60°C ↓ the residue was reconsituted in solvent 100 µl ↓ the solution was injected into HPLC	Results were shown in Table 4.
MTX 1 mcg/serum 1 ml + MeOH 2 ml ↓ centrifuged the supernatant was evaporated to dryness at 60°C ↓	Recovery of MTX was 1.52% (Figure 20).

Table 3 (cont.). Extractions and results of procedures to find serum methotrexate level (condition III).

Extractions	Results
the residue was reconstituted in 0.005 M KH_2PO_4 100 µl ↓ the solution was extracted with ethyl acetate/isopropanol (10/1) 2 ml ↓ the residue was reconstituted in 0.005 M KH_2PO_4 100 µl ↓ the solution was injected into HPLC	

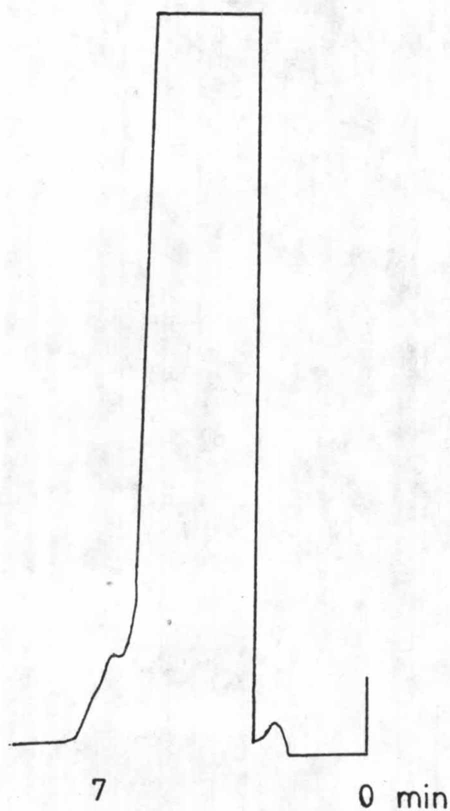


Figure 7. Chromatogram of methotrexate in condition of which applied from Watson, Cohen, and Chen (condition III). Acetonitrile was precipitating agent.

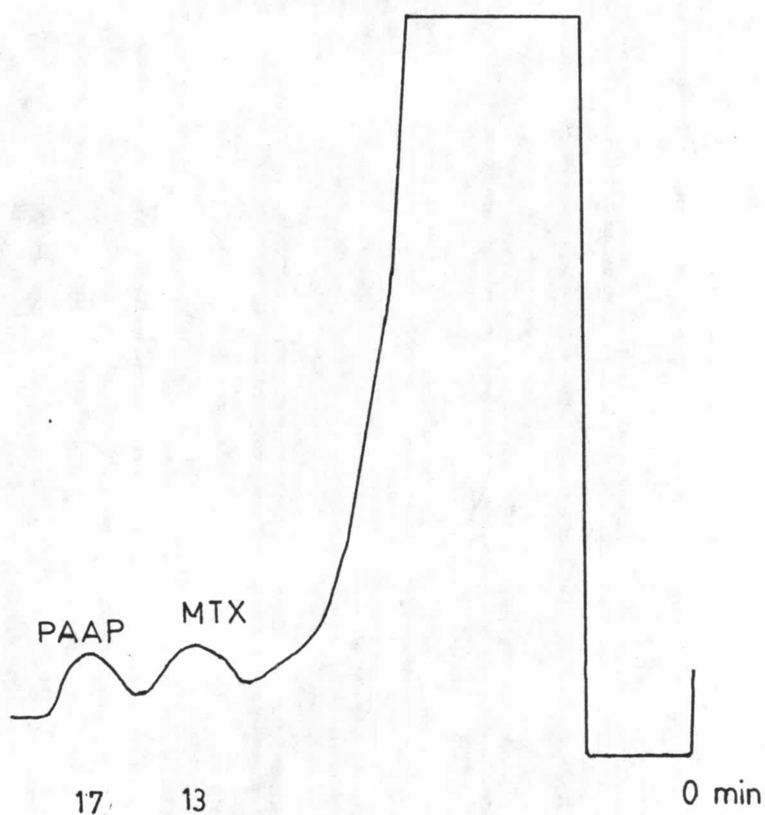


Figure 8. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by acetonitrile and evaporated to concentrate methotrexate concentration.

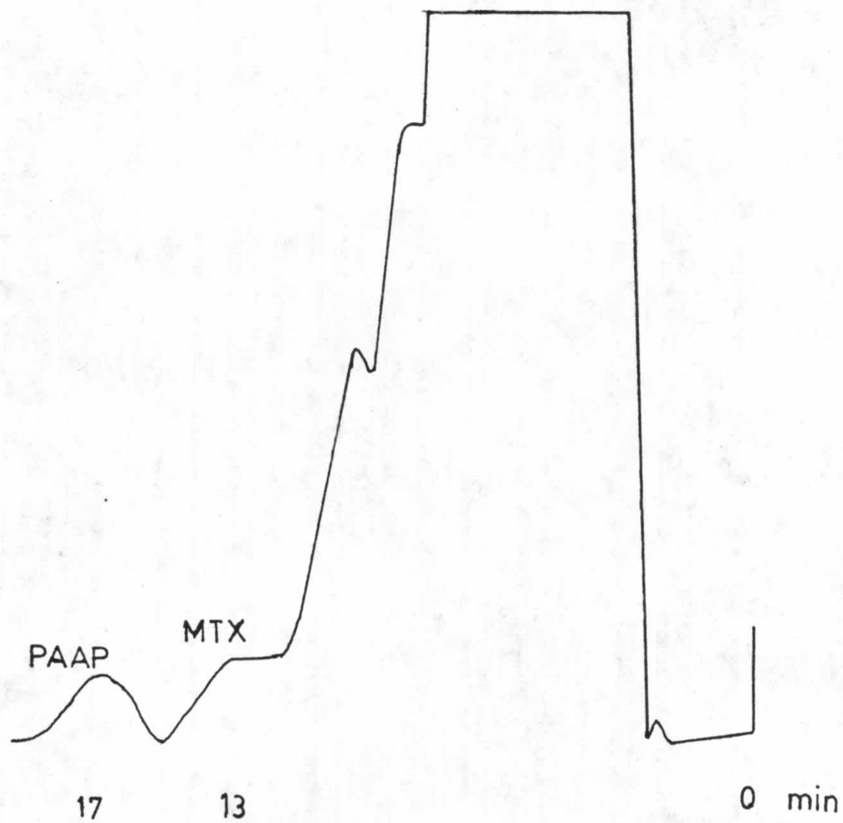


Figure 9. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by methanol and evaporated to concentrate methotrexate concentration.

Table 4. Extractions and results of method to find serum methotrexate level by extraction with organic solvent from Table 3.

Extractions				Results
precipitating agents	pH adjusting agents	organic solvents	solvents	
10 % TCA 0.3 ml	1 N NaOH adjust to pH 6.8	ethyl acetate/ isopropanol =10/1	0.25 M phosphate buffer pH 6.8/ MeOH = 77/23	Recovery of MTX was 29.7% (Figure 10).
10% TCA 0.3 ml		ethyl acetate/ isopropanol = 10/1	0.25 M phosphate buffer pH 6.8/ MeOH = 77/23	RT of MTX was varied. There was interfered peak (Figure 11).
2 N $HClO_4$ 0.6 ml	1 N NaOH	ethyl acetate/ isopropanol = 10/1	0.25 M phosphate buffer pH 6.8/ MeOH = 77/23	Recovery of MTX was 28.7%. Peak of MTX was short (Figure 12).
2 N $HClO_4$ 0.6 ml	1 N NaOH	$CHCl_3$	0.25 M phosphate buffer pH 6.8/ MeOH = 77/23	$CHCl_3$ could not extract MTX and PAAP from serum (Figure 13).

Table 4 (cont.). Extractions and results from Table 3.

Extractions				Results
precipitating agents	pH adjusting agents	organic solvents	solvents	
2 N $HClO_4$ 0.6 ml	1 N NaOH	$CHCl_3$/ isopropanol = 10/1	0.25 M phosphate buffer pH 6.8/ MeOH = 77/23	$CHCl_3$/ isopropanol could not extract MTX and PAAP from serum (Figure 14).
6% $HClO_4$ 1 ml	excess Na_2CO_3	ethyl acetate/ isopropanol = 10/1	0.005 M K_2HPO_4	Serum band was broad (Figure 15).
6% $HClO_4$ 1 ml	5 g of $(NH_4)_2SO_4$	ethyl acetate/ isopropanol = 10/1	0.005 M K_2HPO_4	Recovery of MTX and PAAP were 7.06% and 30% (Figure 16).
6% $HClO_4$ 1 ml		ethyl acetate/ isopropanol = 10/1	0.005 M K_2HPO_4	The organic phase could not be seperated from water phase after MTX extraction.

Table 4 (cont.). Extractions and results from Table 3.

Extractions				Results
precipitating agents	pH adjusting agents	organic solvents	solvents	
acetonitrile 2 ml		ethyl acetate	0.005 M K_2HPO_4	Recovery of MTX was 23.53%. (Figure 17)
acetonitrile 2 ml		ethyl acetate/ isopropanol = 10/1	0.005 M K_2HPO_4	Recovery of MTX was 11.47%. (Figure 18)
acetonitrile 2 ml		$CHCl_3$/ isopropanol = 10/1	0.005 M K_2HPO_4	$CHCl_3$/ isopropanol could not extract MTX from serum. (Figure 19)

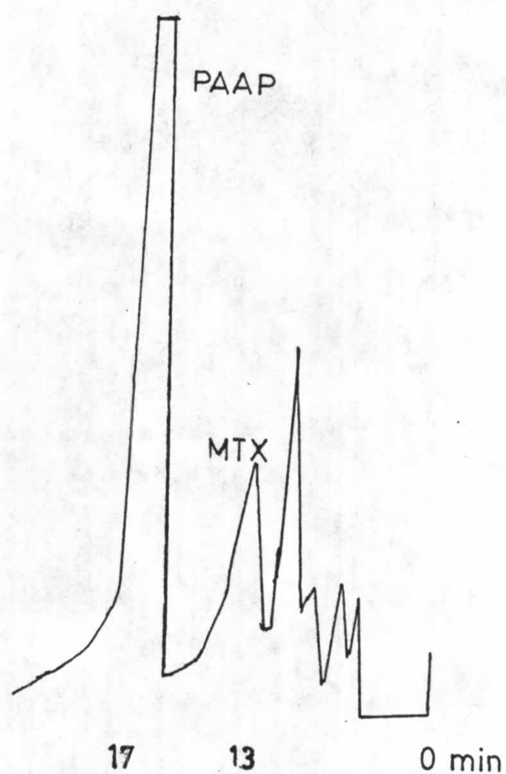


Figure 10. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by trichloroacetic acid, adjusted pH, and extracted.

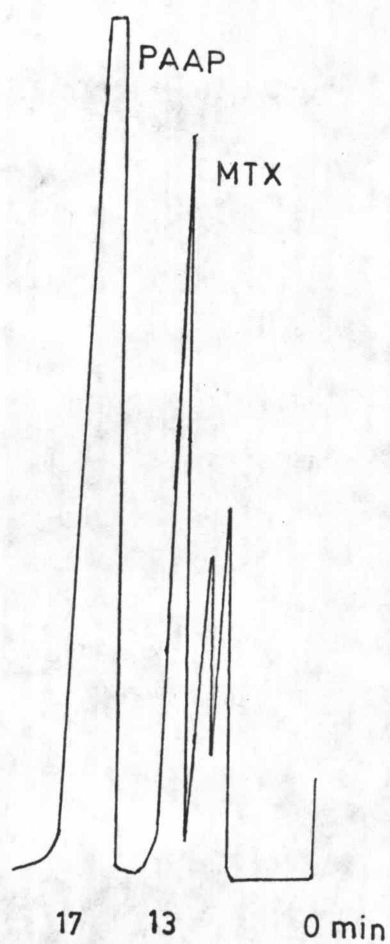


Figure 11. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by trichloroacetic acid and extracted.

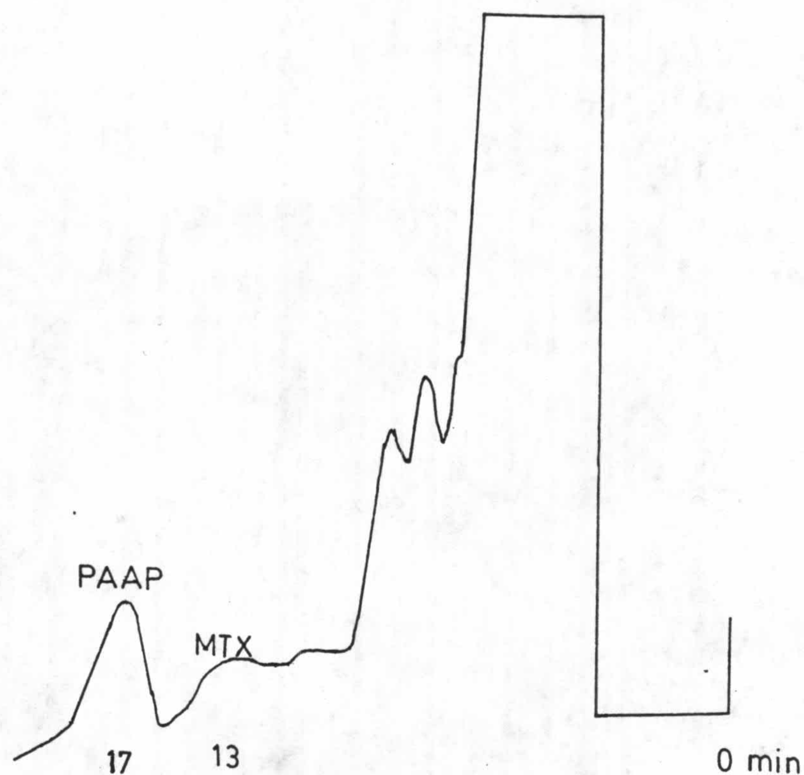


Figure 12. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by perchloric acid, adjusted pH by NaOH, and extracted by ethyl acetate/isopropanol.

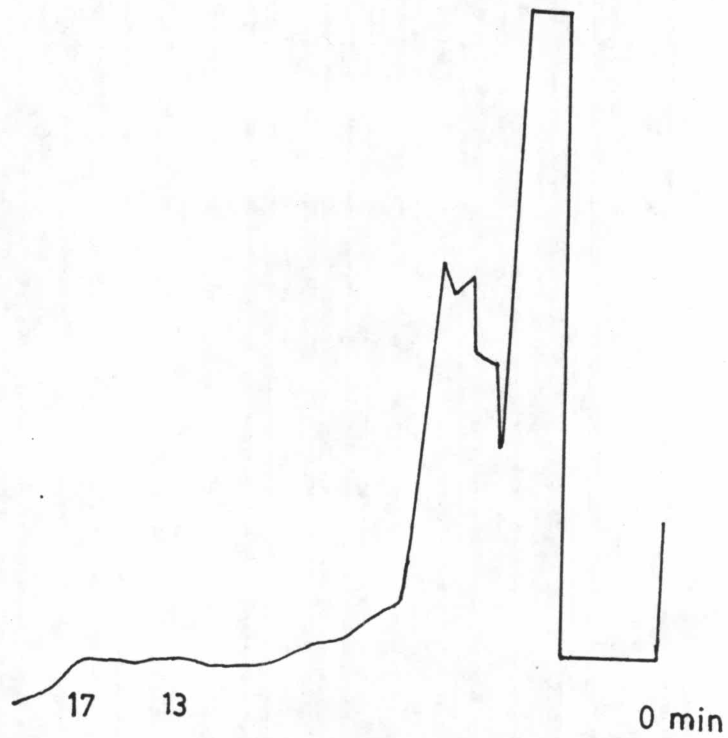


Figure 13. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by perchloric acid, adjusted pH by NaOH, and extracted by chloroform.

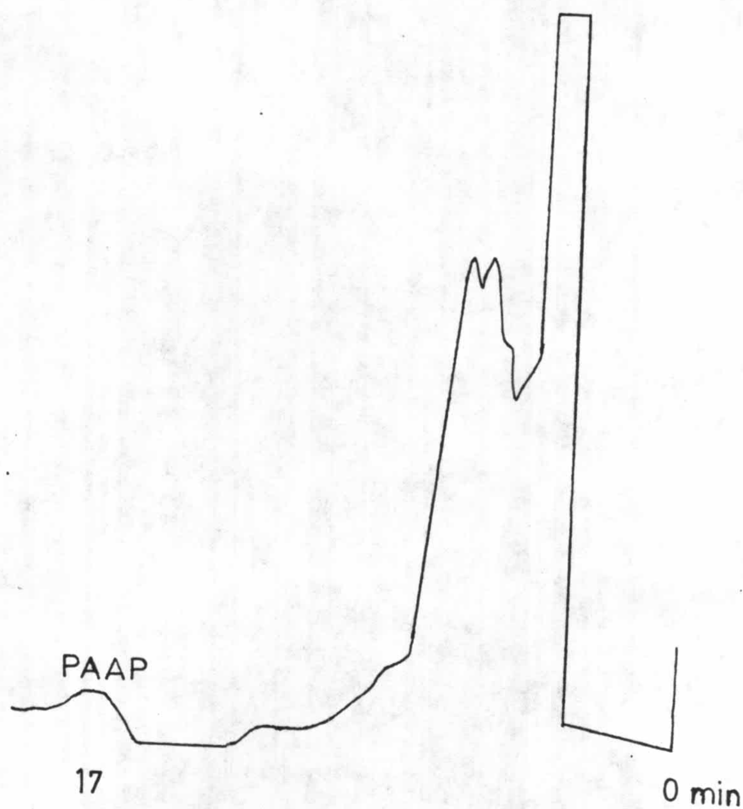


Figure 14. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by perchloric acid, adjusted pH by NaOH, and extracted by $CHCl_3$/isopropanol.

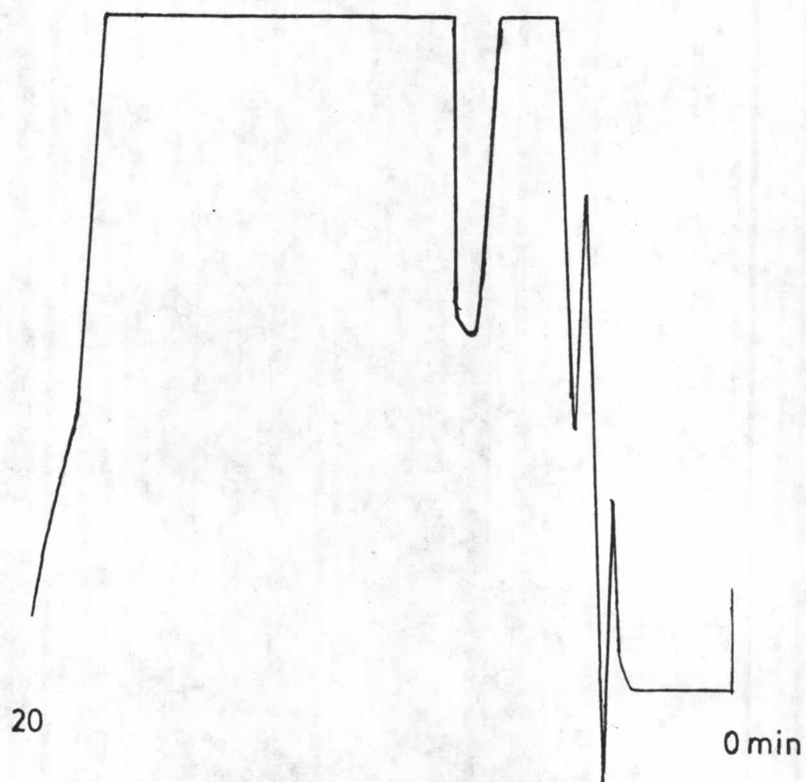


Figure 15. Chromatogram of methotrexate in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by perchloric acid , adjusted pH by Na_2CO_3, and extracted.

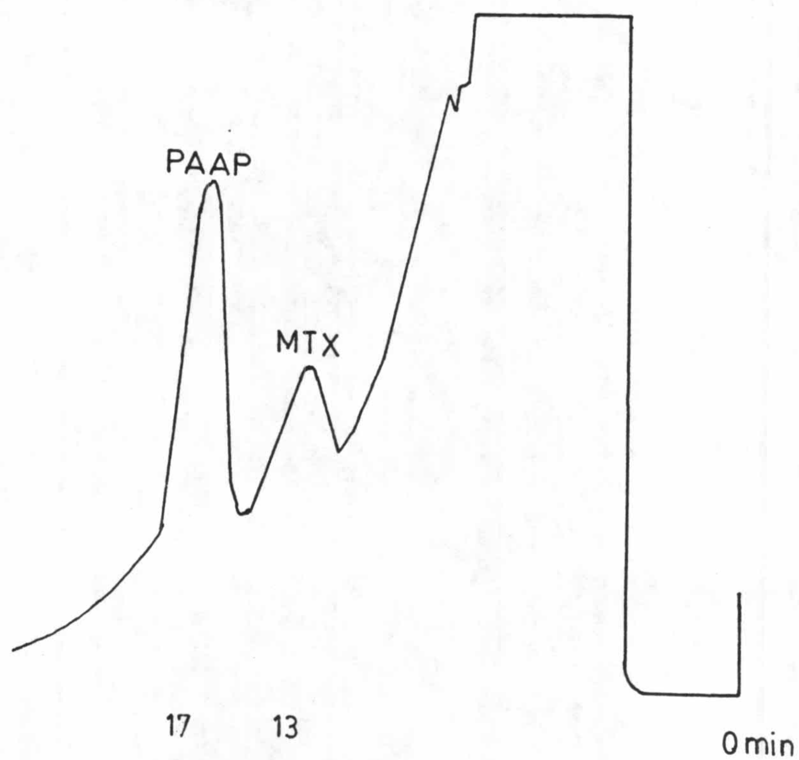


Figure 16. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by perchloric acid , adjusted pH by $(NH_4)_2SO_4$ and extracted.

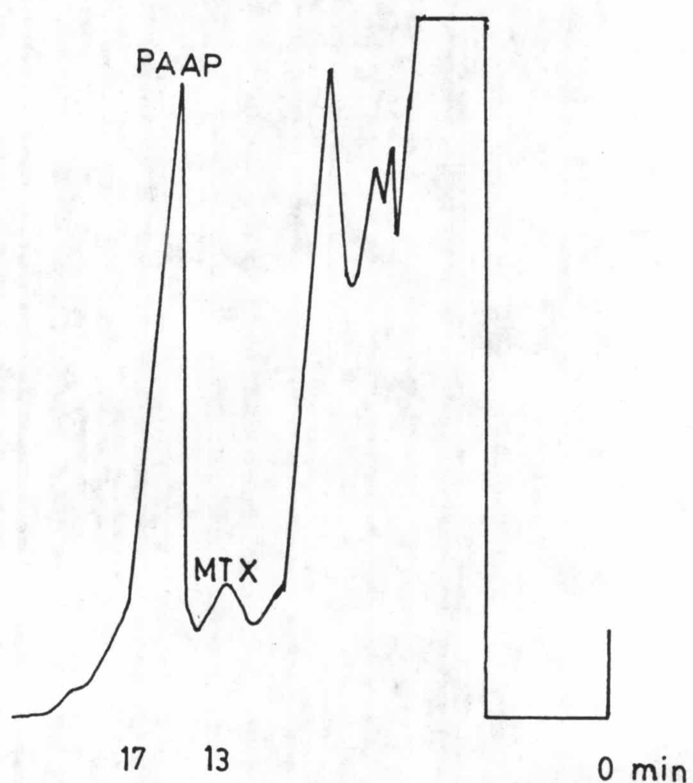


Figure 17. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by acetonitrile and extracted by ethyl acetate.

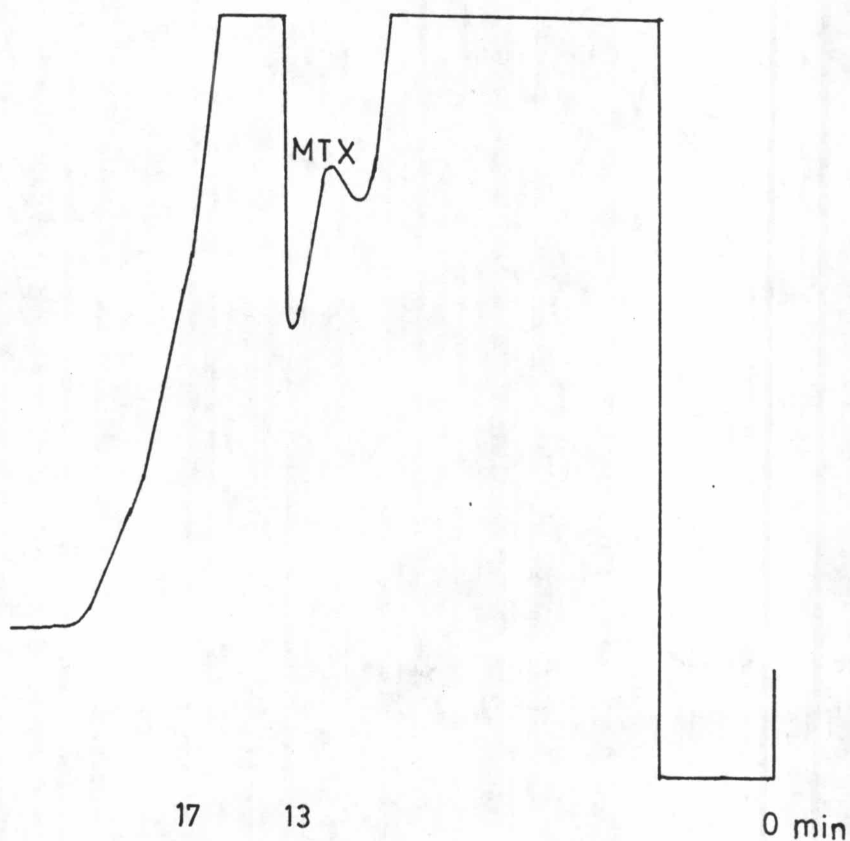


Figure 18. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by acetonitrile and extracted by ethyl acetate/isopropanol.

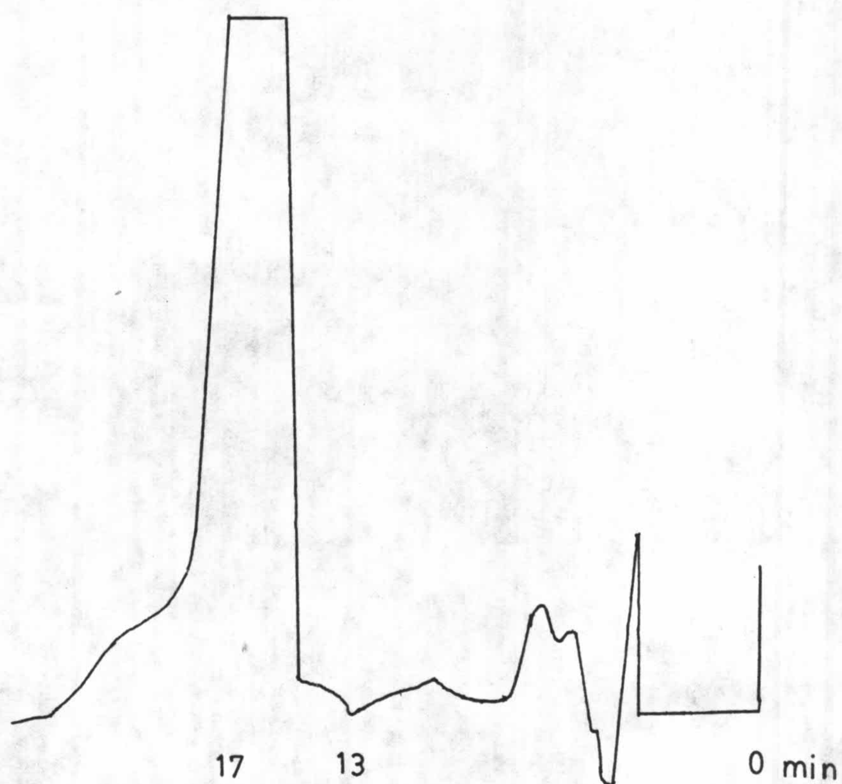


Figure 19. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by acetonitrile and extracted by $CHCl_3$ / isopropanol

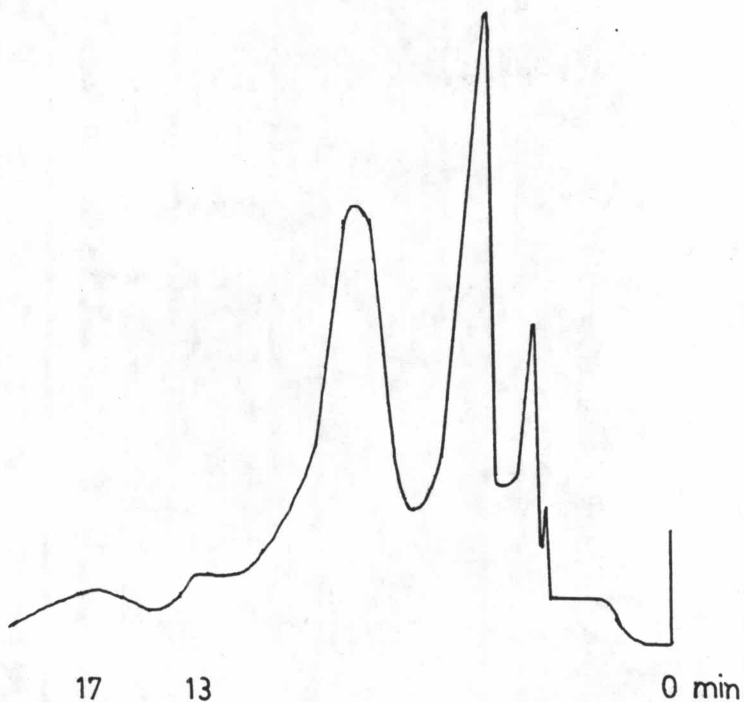


Figure 20. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition III) when serum was precipitated by methanol and double extracted.

residue was reconstituted in phosphate solution. Serum methotrexate was re-extracted to reduce serum band width but the recovery of methotrexate was very low (1.52%). The chromatogram was shown in Figure 20.

1.1.4 <u>Condition IV.</u>

column : radial μ Bondapak C18

detector : ultraviolet spectrophotometer, at 280 nm

flow rate: 1 ml/min

solvent : 0.1 M phosphate buffer pH 6.8/MeOH = 78/22

tested IS: p-aminoacetophenone

Extractions and results were shown in Table 5 and Figures 21, 22. When acetonitrile was used to precipitated serum protein, methotrexate band could not be detected since acetonitrile diluted methotrexate concentration (Figure 21).

Serum protein was precipitated with $HClO_4$, then pH of the supernatant was adjusted with excess $(NH_4)_2SO_4$, next the supernatant was extracted with ethyl acetate/isopropanol, after that the organic phase was evaporated to dryness and the residue was reconstituted in phosphate solution. Retention time of methotrexate and p-aminoacetophenone were 11 and 13 minutes, respectively. Recoveries of methotrexate and p-aminoacetophenone were 39.51% and 52.97%, respectively, and sensitivity for methotrexate was 1.5 mcg/serum 1 ml. The chromatogram was shown in Figure 22. This condition

Table 5. Extractions and results of procedures to fine serum MTX level of which applied from Watson, Cohen and Chen (condition IV).

Extractions	Results
MTX 8 mcg/serum 1 ml + acetonitrile 2 ml ↓ centrifuged the supernatant was injected into HPLC	MTX band could not be detected (Figure 21).
MTX 8 mcg and PAAP 3 mcg/serum 1 ml +1 M $HClO_4$ 1.5 ml ↓ centrifuged $(NH_4)_2SO_4$ 5 g was added to the supernatant ↓ the supernatant was extracted with ethyl acetate/isopropanol (10/1) 2 ml ↓ the organic was evaporated solvent to dryness at 60°C ↓ the residue was reconstituted in 0.005 M K_2HPO_4 100 µl ↓ the solution was injected into HPLC	Recovery of MTX and PAAP were 39.51 and 52.97%, respectively, and sensitivity for MTX was 1.5 mcg/ml (Figure 22).

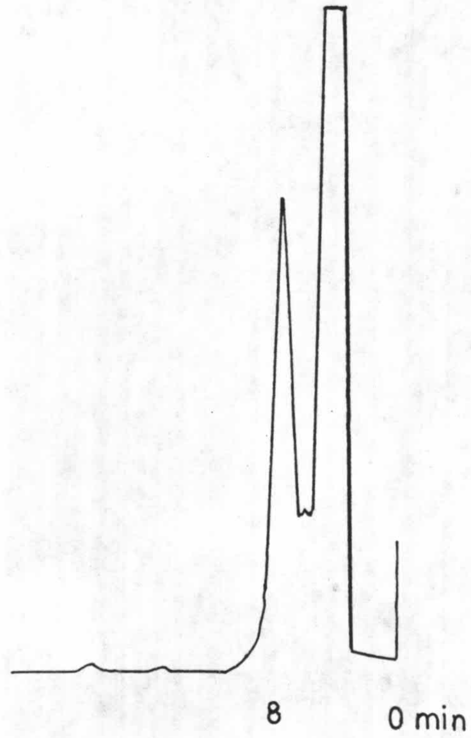


Figure 21. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition IV) when serum was precipitated by acetonitrile.

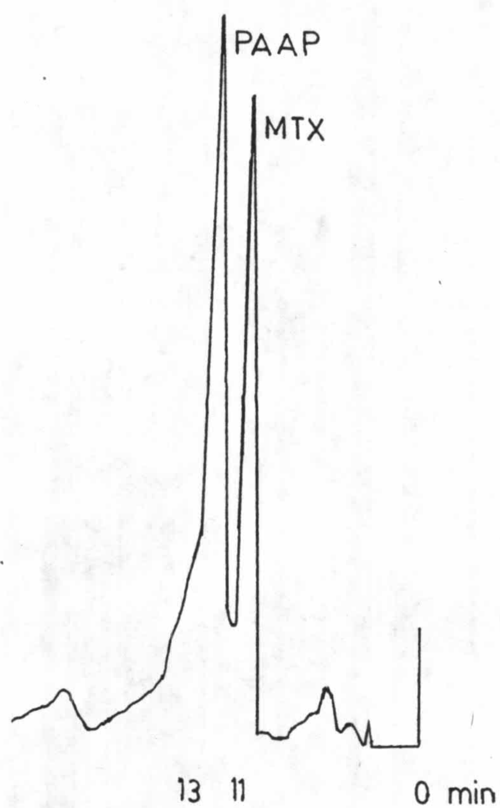


Figure 22. Chromatogram of methotrexate and p-aminoacetophenone in condition of which applied from Watson, Cohen, and Chen (condition IV) when serum was precipitated by perchloric acid, adjusted pH by $(NH_4)_2SO_4$ and extracted.

could use in quantitative analysis for high dose methotrexate administration.

1.2 Using the Condition of which Applied from Lawson (Lawson et al., 1981)

column : radial μ Bondapak C18

detector : ultraviolet spectrophotometer, at 280 nm

flow rate : 1 ml/min

solvent : 0.1 M tris-NaH_2PO_4 pH 6.7/MeOH = 80/20

tested IS : p-aminoacetophenone

extraction: MTX 0.1 mcg in serum 1 ml

+ 2 M $HClO_4$ 0.6 ml

↓ centrifuged

the supernatant was injected into HPLC system

result : Serum band was broad and obscured methotrexate band (Figure 23).

1.3 Using the Condition of which Applied from Canfell (Canfell et al., 1980).

column : radial μ Bondapak C18

detector : ultraviolet spectrophotometer, at 280 nm

flow rate : 1 ml/min

solvent : 0.15 M sodium acetate pH 4.6/acetonitrile = 89/11

tested IS : p-aminoacetophenone, or trimethoprim (TMP)

extraction: MTX 4 mcg in serum 1 ml + acetonitrile 2 ml

↓

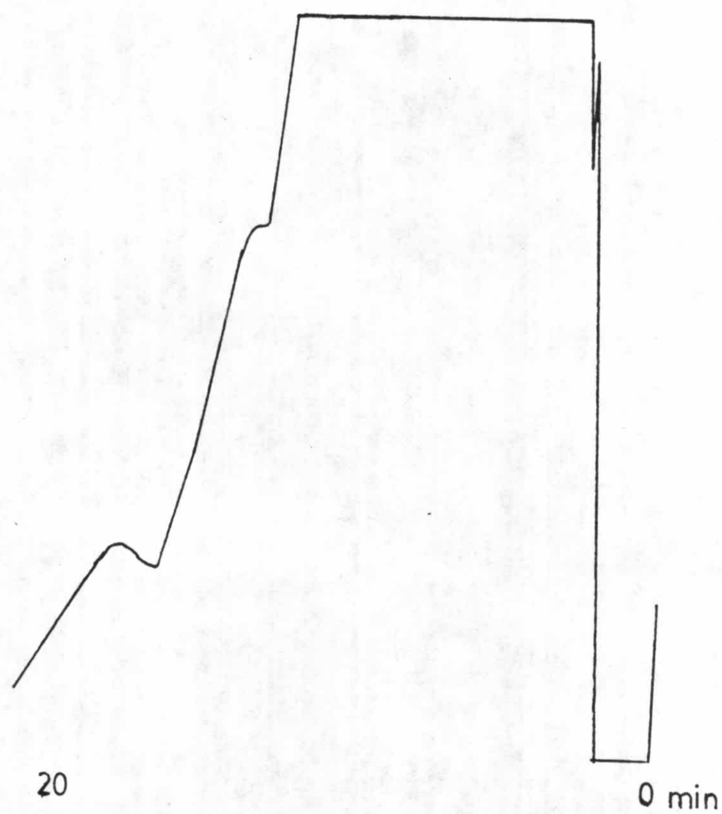


Figure 23. Chromatogram of methotrexate in condition of which applied from Lawson.

↓ centrifuged

the supernatant was extracted with anhydrous ethyl ether and n-butanol for 2 mins

↓ centrifuged

the organic phase was discarded

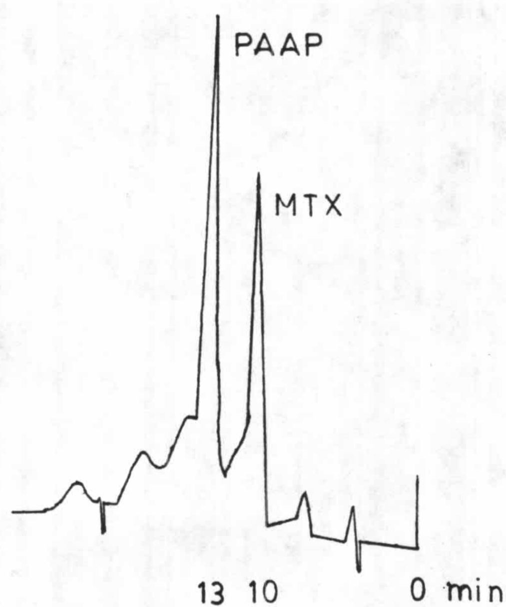
↓

water phase + anhydrous ethyl ether

shaked ↓ centrifuged

the water phase was injected into HPLC system

result : Result of determination of suitable internal standard was shown in Table 6, and Figure 24. Compared retention time and bands of p-aminoacetophenone and trimethoprim, retention time of trimethomeprim was long (21 mins), so it took much time to determine serum methotrexate concentration. Retention time of p-aminoacetophenone was close to retention time of methotrexate. Bands of p-aminoacetophenone and trimethoprim did not interfere the quantitative analysis of methotrexate. p-Aminoacetophenine was not internal standard in this condition since p-aminoacetophenone was extracted by the organic solvents (ethyl ether and n-butanol) and was very little left on water phase. So trimethoprim was internal standard in this condition.



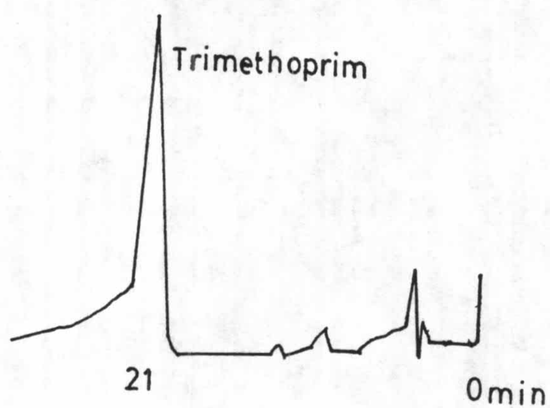


Figure 24. Chromatogram of p-aminoacetophenone and trimethoprim as internal standard in condition of which applied from Canfell.

Table 6. Results of determination of suitable internal standard in condition of which applied from Canfell.

Internal standards	Retention time (minutes)	Notice
PAAP	13	RT of MTX was 10 minutes.
trimethoprim	21	

Amount of the reagents which were used in extraction and the results were shown in Table 7. Serum protein was precipitated, then the clear solution was extracted with 5.5 ml of ethyl ether and 2.9 ml of n-butanol, the water and organic phases became one phase. So the organic phase could not be discarded. When serum was extracted with 5 ml of ethyl ether or 5 ml of ethyl ether and 0.5 ml of n-butanol, the serum band interfered methotrexate band (Figure 25, 26).

All these extractions were not the good one because ethyl ether and n-butanol absorbed water from serum so as to concentrate serum methotrexate. In the air, ethyl ether and n-butanol were quickly evaporated at room temperature so the volume of these organic solvents were inaccurately measured. The serum volume which was little, was inaccurately absorbed by ethyl

Table 7. Extractions and results of procedure to find serum MTX level of which applied from Canfell.

Extractions		Results
anh. ethyl ether (ml)	n-butanol (ml)	
5.5	2.9	When adding 5.5 ml of anh. ethyl ether and 2.9 ml of n-butanol to the supernatant, the water phase and the organic phase were mixed into one phase.
5	-	Serum band interfered MTX band. Sensitivity for MTX was 2 mcg/ml. Recovery of MTX and trimethoprim were 92.42% and 23.61%, respectively. (Figure 25)
5	0.5	Sensitivity for MTX was 16 mcg/ml. Recovery of MTX and trimethoprim were 130% and 20.92%, respectively. (Figure 26)

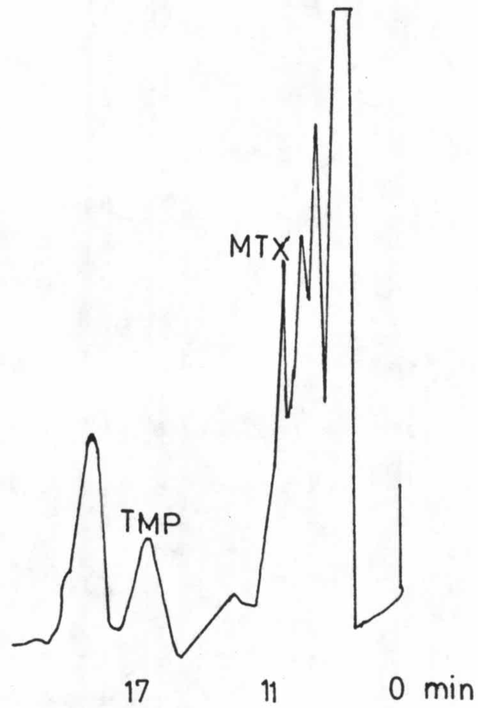


Figure 25. Chromatogram of methotrexate and trimethoprim in condition of which applied from Canfell when serum was extracted with ethyl ether.

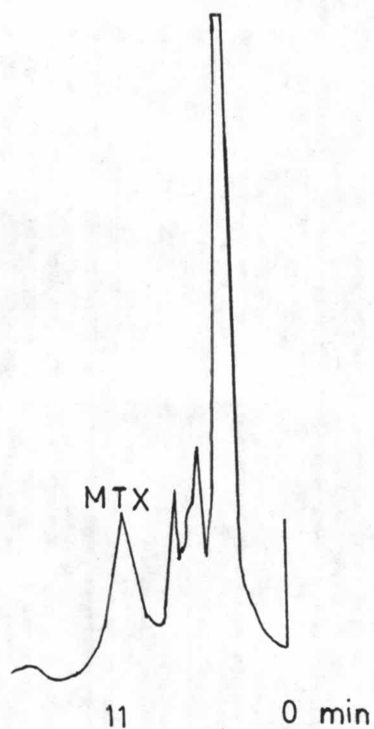


Figure 26. Chromatogram of methotrexate in condition of which applied from Canfell when serum was extracted by ethyl ether and n-butanol.

ether and n-butanol. Serum methotrexate concentrations were varied in range of ±18.9%. Trimethoprim was extracted by ethyl ether and n-butanol unproportional to methotrexate, so in this condition trimethoprim should not be internal standard. If a suitable internal standard could be found, this method would have no variation in serum methotrexate concentration obtained, and would be a good procedure to determine serum methotrexate concentration with high sensitivity.

1.4 <u>Using the Condition of which Applied from Howell</u> (Howell et al., 1980).

column : radial μ Bondapak C18

detector : ultraviolet spectrophotometer, at 280 nm

flow rate : 1 ml/min

solvent : 0.005 M hexanesulfonic acid pH 3.75/MeOH = 74/26

tested IS : p-aminoacetophenone

result : Retention time of p-aminoacetophenone and methotrexate were 10, and 9.5 to 13.5 mins, respectively. Retention time of methotrexate was varied, probably because the hexanesulfonic acid buffer was not stable, weak ionic strength. So this condition was not suiltable to find good internal standard.

1.5 Using the Condition of which Applied from Collier (Collier et al., 1982).

column : radial μ Bondapak C18

detector : ultraviolet spectrophotometer, at 303 nm

flow rate : 0.8 ml/min

solvent : 0.01 M KH_2PO_4 pH 4.5/acetonitrile = 82/18

tested IS : 8-chlorotheophylline (8-CT)

extraction: Sep pak was wash with 10 ml of MeOH, and 10 ml of 0.2 M acetate buffer pH 5.0

↓

100-500 μl of serum and 12.5 mcg of IS were added to 5 ml of 0.2 M acetate buffer pH 5.0, then the solution was applied to Sep pak

↓

Sep pak was washed with 10 ml of distilled water

↓

2 ml of MeOH was applied to Sep pak and elute was collected

↓

the elute was evaporated to dryness at 60°C

↓

the residue was reconstituted in 200 μl of 0.005 M HCl

mixed ↓ centrifuged

75 μl of the soluion was injected into HPLC system

result : The chromatogram of methotrexate and

8-chlorotheophylline standards was shown in Figure 27. Retention times of methotrexate and 8-chlorotheophylline were 8, and 10 mins, respectively. 8-Chlorotheophylline could be internal standard with good resolution.

Sensitivity for methotrexate was 0.1768 mcg/ serum 1 ml. Recovery of methotrexate was 40 %. The chromatogram (Figure 28) had very narrow serum band or some had not, no interferences. Retention time of 8-chlorotheophylline (10 minutes) was close to retention time of methotrexate (8 minutes).

From all experiments, the good procedure to find serum methotrexate concentration was that which applied from the method of Collier. Small volume of serum was required. Sep pak would reduce the serum band and concentrate serum methotrexate. It was easy, and rapid to determine serum methotrexate concentration by applying the serum through the Sep pak without all deproteinizing and extraction procedures. Sep pak was expensive, so it was tried to reuse. Used Sep paks were washed with 25 ml of MeOH, 75 ml of distilled water, 25 ml of MeOH and 25 ml of distilled water, respectively. Methotrexate standards (2.652, 4.420, and 6.188 mcg/ serum 1 ml) were applied through washed and unwashed Sep paks following the method of which applied from Collier. The result was shown in Table 8. There was no significant difference between standard methotrexate

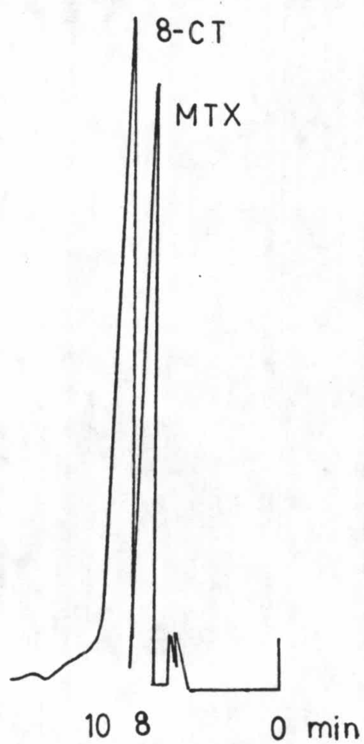


Figure 27. Chromatogram of 8-chlorotheophylline (8-CT) as internal standard in condition of which applied from Collier.

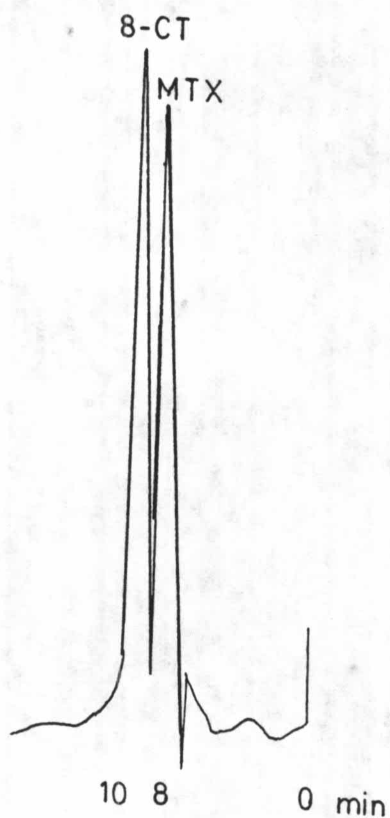


Figure 28. Chromatogram of methotrexate and 8-chlorotheophylline in condition of which applied from Collier when serum was clean up with Sep pak.

Table 8. Comparing standard methotrexate concentrations with serum methotrexate concentrations obtained from washed and unwashed Sep paks.

MTX standards (mcg/ml)	Serum MTX concentrations obtained (mcg/ml)	
	Washed Sep paks	Unwashed Sep paks
2.652	2.6952	3.2513
4.420	4.3847	5.4117
6.188	6.1138	7.5741

concentrations and serum methotrexate concentrations that were applied through washed Sep paks ($p>0.1$). But serum methotrexate concentrations which obtained from applying the serum through unwashed Sep pak were significantly different from standard methotrexate concentrations ($p<0.025$) [Appendix E]. Alhough washed Sep paks could be reused to save money but guard column might easy be dirty. Other solvents should be found to wash used Sep paks in order to clearly remove serum protein.

In Vivo Studies

1. Subjects.

Physiological characteristics and biochemical laboratory results of the patients were shown in Appendix B.

Biochemical laboratory results of almost all patients were within the normal limit (Table 14). But patients number 4 and 6 might be diabetes mellitus. Serum creatinine of patient number 11 was lower than the normal limit. Serum albumin of patient number 5 was lower than the normal value, so as to patients number 6, and 11. Besides these, total serum protein of patient number 6 whose age was 70 years, was lower than the normal value. Patients number 3, 4, and 9 had higher alkaline phosphatase than the normal limit, their liver functions were good. Patient number 7 had much high total bilirubin and low hematocrit.

2. Assay for Methotrexate in Serum.

Typical chromatogram from serum containing both methotrexate and internal standard was shown in Figure 28. Retention times of methotrexate and 8-chlorotheophylline were 8 and 10 mins, respectively. Chromatographic response was readily for serum methotrexate concentrations ranged from 0.1768 to 8.840 mcg/ml (see Appendix F, and Figure 29). The reproducibility of the method was tested using multiple replication (n = 5), the coefficient of variation was 2.59 at the highest concentration and 7.61 at the lowest concentration. The analytical procedure was highly specific and reproducible. The sensitivity of methotrexate detection in human serum was 0.1768 mcg/ml.

3. Serum Methotrexate Level.

The individual serum methotrexate concentrations at each appropriate sampling time from 0 to 12 hours were shown in Table 9 (see Appendix G for their semilogarithmic plots). The average values were illustrated graphically in Figure 31.

4. Pharmacokinetic Analysis.

Based on a semilogarithmic plots of individual serum concentration-time data for 11 patients. Using the method of residuals (Gibaldi, and Perrier, 1982b), the initial estimates of pharmacokinetic parameters were obtained. The experimental data and the initial estimates of the parameters were tried to fit the models in PCNONLIN nonlinear estimation program on a digital computer. The goodness of fits for each model were obtained by comparing the values of individual sum of squares of the deviations between experimental data and calculated values. Results showed that two-compartment model with bolus input and first-order output (Model 8) had a better fit with the least sum of squares of the deviations. To fit to the Model 8, the average correlation coefficient between experimental data and calculated values observed for individual serum level data was 0.998 ± 0.001 (Appendix H).

The relevant pharmacokinetic parameters calculated by PCNONLIN were shown in Table 10. The

Table 9. Individual serum MTX concentration from 11 subjects following intravenous administration of MTX 1 mg/kg of body weight.

Time (hr)	Serum MTX concentration (mcg/ml) from Subject 1	2	3	4	5	6	7	8	9	10	11	Mean ±SEM
0.167	3.4949	5.4183	6.2989	6.1966	6.1029	2.8405	4.1213	2.9720	2.7744	4.3012	3.7063	4.3752 ±0.4192
0.5	2.6064	3.1496	4.3377	4.0760	3.7481	1.8366	2.1121	1.5551	1.9102	2.3762	1.7494	2.6779 ±0.3002
1	1.8484	2.0132	2.7119	3.1914	2.7096	1.3976	1.1694	1.2151	1.2131	1.7363	1.4186	1.8750 ±0.7054
2	1.2683	1.5636	2.2654	3.1065	1.9665	1.2308	0.9477	1.0694	0.9619	1.4729	1.1398	1.5448 ±0.2127
4	1.2253	1.4429	1.2259	1.7955	1.3548	0.8790	0.6054	0.3755	0.4558	1.0714	0.6840	1.0105 ±0.1354
6	0.9040	1.0835	0.9457	1.6232	1.0980	0.4117	0.3019	0.2927	0.3117	0.7547	0.4328	0.7418 ±0.1305
8	0.6402	0.7655	0.4127	0.8754	0.6488	0.2509	0.2147	0.2345	0.2509	0.6182	0.4180	0.4845 ±0.0710
12	0.5202	0.6998	0.3607	0.6541	0.4887	0^+	0^+	0^+	0^+	0.4702	0.3667	–

0^+ = could not detect serum MTX by the method which was used in the study.

average peak of serum methotrexate concentration, and theoretical drug concentrations at t_0 extrapolated from the distribution line and elimination line obtained after single intravenous dose of methotrexate 1 mg/kg of body weight were 6.7449±0.5741, 4.6381±0.4305, and 2.1068 ±0.2460 mcg/ml, respectively. The average distribution and elimination rate constant were 4.1500±0.5865 and 0.1861±0.0192 hr^{-1}, respectively. The average distribution and elimination half lifes of methotrexate were 0.2005±0.0262 and 4.2301±0.5214 hours, respectively. Area under the serum concentration-time curve and volume of central distribution were 14.0580±1.9858 mcg/ml.hr and 8.1560±0.9237 L. Pharmacokinetic equation for calculating serum methotrexate concentration was as follow:

$$C_t = 4.6381e^{-4.15t} + 2.1068e^{-0.1861t}$$

The average volume of distribution and total clearance calculated according to equation 3 and 4 were 23.7068±2.2931 L and 4.4888±0.7449 L/hr, respectively (Table 11).

For methotrexate, two-, or three- compartment models had been described (Breithaupt, and Kuenzlen, 1982; Azarnoff, Wan, and Huffman, 1974; Lokiec, Poirier, Gisselbrecht, Marty, Boiron, and Najean, 1982; Stewart et al., 1985). From two-compartment modeling, α and β

Table 10. Compartment modeling pharmacokinetic parameters from 11 subjects following IV administration of 1 mg/kg of MTX using PCNONLIN.

Parameter	Subject											Mean
	1	2	3	4	5	6	7	8	9	10	11	±SEM
Peak serum concentration at t_0, C_0 (mcg/ml)	4.2416 ±0.4187	7.7360 ±0.4601	8.2200 ±1.0125	9.5467 ±2.3402	8.3760 ±0.4924	4.4376 ±1.1439	6.4810 ±0.4374	7.1811 ±6.1726	3.5807 ±0.5015	6.7988 ±0.4421	7.5944 ±1.9006	6.7449 ±0.5741
Theoretical drug concentration at t_0 extrapolated from the distribution phase, A (mcg/ml)	2.6659 ±0.2285	5.8272 ±0.3190	5.0638 ±0.5414	5.6199 ±2.0089	5.6245 ±0.3110	2.6125 ±0.9663	5.0881 ±0.2868	5.4732 ±5.9505	2.1653 ±0.2491	4.9098 ±0.3492	5.9686 ±1.7322	4.6381 ±0.4305
Theoretical drug concentration at t_0 extrapolated from the elimination phase, B (mcg/ml)	1.5757 ±0.1903	1.9088 ±0.1411	3.1562 ±0.4712	3.9268 ±0.3313	2.7514 ±0.1814	1.8251 ±0.1775	1.3929 ±0.1506	1.7079 ±0.2221	1.4154 ±0.2524	1.8890 ±0.0929	1.6258 ±0.1685	2.1068 ±0.2460
Distribution rate constant, α (hr^{-1})	1.8388 ±0.3761	2.9805 ±0.3241	2.5942 ±0.6814	5.1446 ±2.4388	2.9877 ±0.3612	5.2752 ±2.6400	3.6071 ±0.4247	8.3937 ±7.1196	2.5034 ±0.7207	4.1523 ±0.4761	6.1722 ±1.9526	4.1500 ±0.5865

Table 10 (cont.). Compartment modeling pharmacokinetic parameters from 11 subjects using PCNONLIN.

Parameter	Subject 1	2	3	4	5	6	7	8	9	10	11	Mean ± SEM
Elimination rate constant, β (hr^{-1})	0.0952 ±0.0207	0.0930 ±0.0142	0.2164 ±0.0393	0.1654 ±0.0234	0.1641 ±0.0159	0.2196 ±0.0334	0.2273 ±0.0340	0.2990 ±0.0578	0.2465 ±0.0507	0.1365 ±0.0118	0.1840 ±0.0316	0.1861 ±0.0192
Distribution half-life, $\alpha t_{\frac{1}{2}}$ (hr)	0.3770 ±0.0770	0.2326 ±0.0253	0.2672 ±0.0701	0.1347 ±0.0638	0.2320 ±0.0280	0.1314 ±0.0657	0.1922 ±0.0226	0.0826 ±0.0700	0.2769 ±0.0796	0.1669 ±0.0191	0.1123 ±0.0355	0.2005 ±0.0262
Serum half-life, $\beta t_{\frac{1}{2}}$ (hr)	7.2811 ±1.5818	7.4533 ±1.1346	3.2025 ±0.5805	4.1917 ±0.5928	4.2235 ±0.4081	3.1560 ±0.4796	3.0494 ±0.4560	2.3186 ±0.4480	2.8117 ±0.5772	5.0771 ±0.4386	3.7664 ±0.6462	4.2301 ±0.5214
Area under the curve, AUC (mcg/ml.hr)	18.0016 ±2.2005	22.4801 ±2.0949	16.5344 ±1.4097	24.8392 ±2.1975	18.6475 ±0.9616	8.8055 ±0.7880	7.5383 ±0.5283	6.3651 ±0.6840	6.6063 ±0.5904	15.0184 ±0.7822	9.8014 ±0.9935	14.0580 ±1.9858
Volume of central distribution, Vc (L)	11.7879 ±0.6252	6.4633 ±0.2968	5.8394 ±0.4210	5.8659 ±1.3379	4.1786 ±0.1779	12.3940 ±2.9713	7.7149 ±0.4070	8.3553 ±7.1005	12.8467 ±0.9197	9.2664 ±0.5288	5.0037 ±1.2045	8.1560 ±0.9237
k_{10} (hr^{-1})	0.2356 ±0.0339	0.3441 ±0.0388	0.4971 ±0.0589	0.3843 ±0.0985	0.4492 ±0.3227	0.5040 ±0.1349	0.8597 ±0.0809	1.1282 ±0.9441	0.5420 ±0.0664	0.4527 ±0.0380	0.7748 ±0.2100	0.5611 ±0.0782
k_{12} (hr^{-1})	0.9554 ±0.1950	1.9239 ±0 2179	1.1841 ±0.3450	2.7122 ±1.6854	1.6110 ±1.2156	2.6919 ±1.7765	2.0210 ±0.2656	5.3403 ±5.9424	1.0693 ±0.3370	2.5838 ±0.3394	4.1155 ±1.5580	2.3826 ±0.4060
k_{21} (hr^{-1})	0.7429 ±0.1963	0.8055 ±0.1102	1.1294 ±0.3548	2.2134 ±0.7278	1.0916 ±0.1506	2.2989 ±0.8121	0.9537 ±0.1555	2.2242 ±0.4295	1.1386 ±0.4086	1.2522 ±0.1324	1.4660 ±0.2736	1.3924 ±0.1757

Table 11. Volume of distribution and total clearance of 11 subjects.

Parameter	Subject 1	2	3	4	5	6	7	8	9	10	11	Mean ±SEM
Volume of distribution, Vd (L)	29.1757	23.9160	13.4152	13.6306	11.4377	28.4431	29.1808	31.5264	28.2477	30.7315	21.0706	23.7068 ±2.2931
Total clearance, Cl_T (L/hr)	2.7775	2.2242	2.9030	2.2545	1.8769	6.2461	6.6328	9.4264	6.9631	4.1948	3.8770	4.4888 ±0.7449

half-life were 0.58, and 2.08 hours, respectively (Breithaupt et al., 1982). From three-compartment modeling, α, β, and γ half-life were 0.75, 3.49, and 26.99 hours, respectively (Azarnoff et al., 1974); 0.46, 1.82, and 6.3 hours, respectively (Lokiec et al., 1982); and 0.3, 3.09, and 33 hours, respectively (Stewart et al., 1985). The final phase (γ phase) probably represented a combined effect of drug release from deep compartments, enterohepatic circulation, plural effusion, and renal tubular reabsorption. In this study, α and β half-life (0.2 and 4.2 hours) were close to those reported by Azarnoff and Stewart (Azarnoff et al., 1974; Stewart et al., 1985). Methotrexate might diffuse to enterohepetatic circulation, plural effusion or have tubular reabsorption in small amount because of low dose methotrexate administration.

Average tolal clearance, 4.49 L/hr, was close to the total clearance which had been reported by Azarnoff and his associates, 4.68 L/hr; and by Breithaupt and his co-workers, 3.71 L/hr (Azarnoff et al., 1974; Breithaupt et al., 1982). The patient creatinine clearance was usually used as an indicator of his or her renal function because this endogenous substance underwent complete glomerular filtration while having very little tubular reabsorption.

Average volume of distribution, 23.71 L, was close to that reported by Lippens, 24.9 L, but lesser

than that reported by Stewart, 37 L (Lippens, 1984; Stewart et al., 1985). Volume of central distribution of methotrexate (Vc) was approximately to extracellular water, which was about 18% of body weight (Stewart et al., 1983). Vc was 9 L in patient whose weight was 50 kgs which was close to the Vc from this study, 8.16 L.

Area under the concentration-time curve (AUC) of patients who had been given methotrexate 1 mg/kg of body weight was varied in the range of 6.37 to 24.84 mcg/ml.hr. The average AUC was 14.06 mcg/ml.hr. AUC was sum of the product of drug concentration and a period of time from t_0 to t_∞. AUC implied drug amount containing in the body.

Average peak serum concentration after taking 1 mg/kg of methotrexate was 6.75 mcg/ml (3.58 - 9.55 mcg/ml). Comparing this with C_0 reported by Lokiec which ranged from 4.09 to 18.63 mcg/ml when giving methotrexate 50 mg/m^2 of body surface area, both C_0 were close to each other since the dose of methotrexate given in tha latter case was a little higher.

The severity of methotrexate toxicity was directly proportional to the duration of methotrexate exposure beyond the threshold time and extracellular methotrexate concentration above the threshold. Bone marrow and epithelium were sensitive to methotrexate and both had threshold time and threshold concentration of

42 hours, and 0.036 mcg/ml, recpectively (Balis et al., 1983). From the pharmacokinetic equation in this study, after intravenous injection of methotrexate 1 mg/kg of body weight for 21.9 hours serum methotrexate concentration would decrease to 0.036 mcg/ml. So this methotrexate should not induce toxicity to patients. However, patients who received higher dose should be monitored the serum methotrexate concentration. If its pharmacokinetic still followed two-compartment open model, and relationship between methotrexate dose and serum methotrexate concentration was linear, serum methotrexate concentrations of patients at 42 hours were calculated using the pharmacokinetic equation as follow:

$$C_t = 4.6381e^{-4.15t} + 2.1068e^{-0.1861t}$$

If their serum methotrexate concentrations were above 0.036 mcg/ml, leucovorin rescues were given to the patients.

5. Evaluation of Clinical Study.

After the therapeutic course of methotrexate subsequent by radiotherapy, tumor size of patients were reduced (Appendix I). Tumor response of 4 patients could not be evaluated. Tumor size of nasopharynx cancer of patients number 1 and 11 could not be measured and primary site of tumor for patient number 10 suffering from metastatic cancer could not be found. Patient number 5 was lost to follow up. 7 Of 11 patients (100%)

responsed to the therapy with complete response of 57.1%, and partial response rate of 42.9% (Table 12). Cancer of tongue completely responsed to the therapy because tumor sizes of the patients before treatment were small or were on the primary state.

Table 12. Clinical responses of patients to the therapeutic course of methotrexate and radiotherapy.

Diagnosis for cancer of	No. of patients	Tumor responses		
		CR	PR	Others
tongue	4	4	-	-
soft palate	1	-	1	-
lower gum	1	-	1	-
pharynx	1	-	1	-
buccal mucosa	1	-	-	NF
metastasis cancer	1	-	-	NM
nasopharynx	2	-	-	NM
total	11	4 (57.1%)	3 (42.9%)	4

CR = complete response, PR = partial response

NF = Patient did not followed up the therapy.

NM = Tumor size could not be measured.