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Note

Simultaneous determination of acetaminophen, theophylline and salicylate in serum by high-performance liquid chromatography

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Determination of acetaminophen, theophylline and salicylate is commonly requested in our laboratory, for studies of both drug therapy and drug intoxication. A method for the simultaneous determination of the three drugs would therefore be very useful.

Many methods for the separate high-performance liquid chromatographic (HPLC) determination of acetaminophen, theophylline and salicylate have been described [1—26]. The simultaneous determination of acetaminophen and salicylate was reported by Miceli et al. [27]. However, theophylline was not resolved from acetaminophen and could therefore interfere.

Preliminary results using isocratic reversed-phase liquid chromatography with radially compressed columns, have indicated that the composition of the mobile phase is critical to obtain resolution of the peaks. A simple pretreatment of the serum sample, e.g. addition of acetonitrile to precipitate proteins, centrifugation and injection of the clear supernatant liquid, was investigated and found not to be applicable since endogenous compounds interfering with the determination of acetaminophen were detected.

EXPERIMENTAL

Apparatus

A Liquid chromatography pump Model 45 (Waters Assoc., Milford, MA, U.S.A.) connected to a UV detector Model 445 (Waters Assoc.) were used for the determinations. The detector was equipped with a 280-nm interference filter. The liquid chromatograph was connected to a Sigma 10 Chromatography Data Station (Perkin-Elmer, Norwalk, CT, U.S.A.).

The HPLC column was a plastic C_{18} column (10 cm × 5 mm I.D.; 10 μm

particle size) fitted into a radial compression module RCM 100 (Waters Assoc.).

The mobile phase was 28% (v/v) of methanol in acetate buffer (pH 3.6). (Buffer preparation: to 2.7 g of sodium acetate in 1000 ml of deionized water were added 15.0 ml of concentrated acetic acid.) The mobile phase flow-rate was 1.0 ml/min.

Reagents

Sodium acetate and concentrated acetic acid were of A.R. grade (Merck, Darmstadt, G.F.R.). Methanol, methylene chloride and isopropanol were of HPLC grade (Baker, Phillipsburg, NJ, U.S.A.)

The internal standard solution was prepared by dissolving 15 mg of 8-chloro-theophylline (Sigma, St. Louis, MO, U.S.A.) in 100 ml of ethanol.

Serum was supplied from the hospital's blood bank. Serum drug standards were prepared by adding known amounts of acetaminophen, theophylline and salicylate to drug-free serum.

The extraction solvent was methylene chloride—isopropanol (90:10).

Procedure

To 200 μl of serum were added 20 μl of the internal standard solution and 200 μl of 1.0 M HCl. After thorough mixing, 2 ml of the extraction solvent were added. The tubes were vortexed for 5 min in a multi-tube vortexer and then centrifuged for 2 min at 2500 g.

The aqueous supernatant layer was aspirated off and discarded. The organic phase was transferred to a new tube with the aid of a pasteur pipette and evaporated to dryness at 50°C with a gentle stream of air.

The residue was dissolved in 100 μl of the mobile phase. The extract was then transferred to a Spinco plastic tube and centrifuged in a hematocrite centrifuge (10,000 g) for 3 min. A 20-μl volume of the clear supernatant liquid was then injected into the chromatograph.

RESULTS

Fig. 1 shows chromatograms obtained with the procedure. Acetaminophen, theopylline, salicylate and 8-chlorotheophylline were all resolved under the chromatographic conditions used. However, salicylate showed minor peak tailing, but this did not seriously affect its quantitative determination.

No major interfering peaks of biogenic origin were detected. Blank values as computed by the Sigma 10 Data Station were as follows: acetaminophen 0.2 μg/ml; theophylline 0.7 μg/ml; salicylate 1.1 μg/ml.

Table I gives the precision data obtained by repeated analysis of a serum to which all the drugs had been added. The following practical detection limits (computed as approximately three times the blank values) were set: acetaminophen 1 μg/ml; theophylline 2 μg/ml; salicylate 3 μg/ml.

No interference to acetaminophen, theophylline or salicylate was found from: hexapropymate, meprobamate, methyprylon, methaqualone, glutethimide ("neutral" drugs); oxazepam, chlordiazepoxide, diazepam (benzodiazepines); phenobarbital, metharbital, heptabarbital, butalbital, secobarbital, aprobarbital (barbiturates); gentamicin, tobramycin, cloxacillin (antibiotics; for
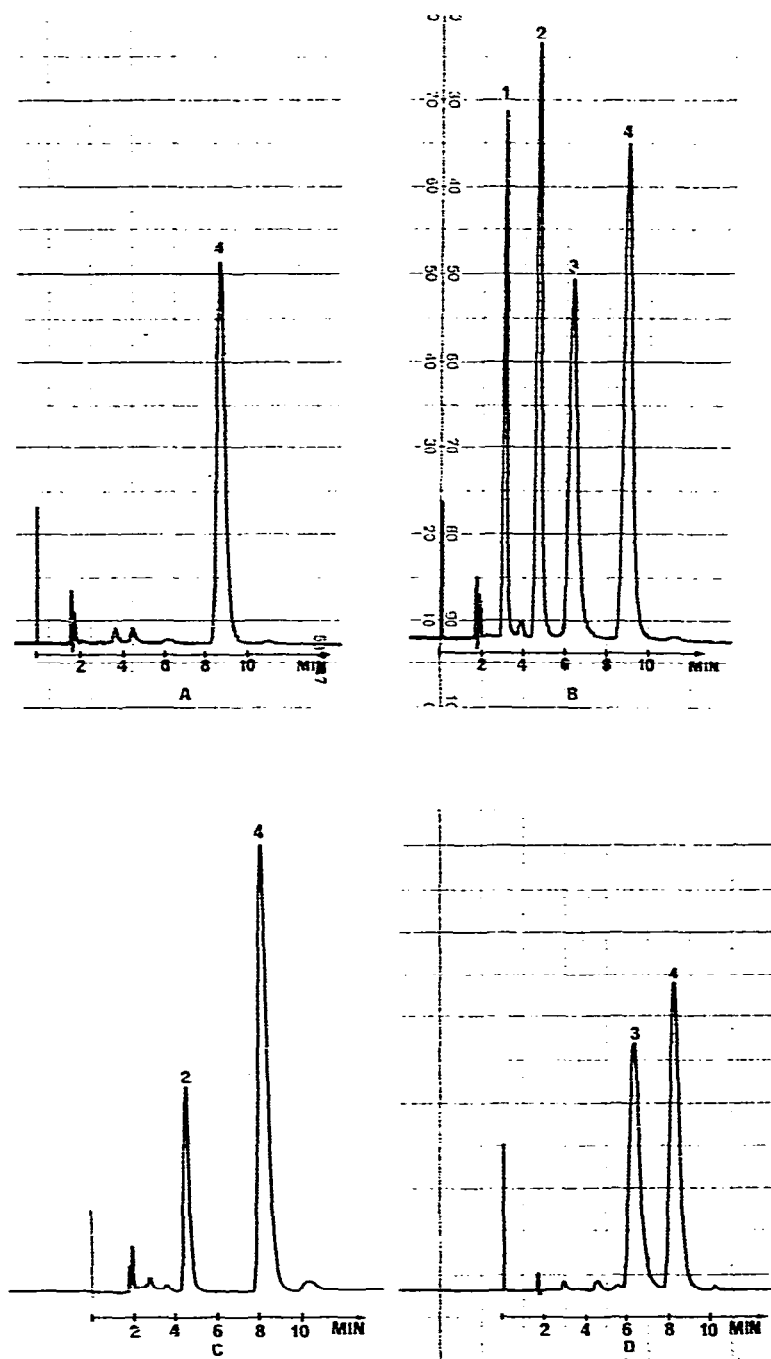
Fig. 1. Chromatogram A: a typical serum blank. Chromatogram B: serum standard. 1 = acetaminophen (100 μg/ml), 2 = theophylline (20 μg/ml), 3 = salicylate (200 μg/ml), and 4 = 8-chlorotheophylline (internal standard). Chromatogram C: sample from a patient on theophylline medication (theophylline concentration = 8.1 μg/ml). Chromatogram D: sample from a patient on salicylate medication (salicylate concentration = 240 μg/ml).

TABLE I

PRECISION DATA

Compound	Mean (μg/ml)	n	C.V. (%)	Added (μg/ml)	Range of linearity* (μg/ml)
Acetaminophen	10.5	8	12.1	12.5	
	24.7	5	3.0	25.0	10—200
	51.0	8	3.4	50.0	
	98.4	8	3.9	100.0	
Theophylline	3.7	8	4.1	3.8	
	7.7	5	3.5	7.5	2—60
	15.2	8	2.3	15.0	
	32.5	8	3.2	30.0	
Salicylate	26.2	8	9.1	25.0	
	97.1	8	5.8	100.0	20—500
	212.4	13	7.2	200.0	

*The calibration graphs of peak area ratios of the drugs and the internal standard versus drug concentration are linear in the given ranges (all correlation coefficients are greater than 0.98). The calibration graphs all pass through the origin at zero drug concentration ("zero intercept").

exception see cefuroxime, Table II); amitriptyline, imipramine, desipramine, nortriptyline, protriptyline, trimipramine (tricyclic antidepressants); phenytoin, ethosuximide, carbamazepine, primidone (antiepileptic drugs); sulfadimethoxine, sulfaisodimidine, sulfaproxylin (sulfonamides; see Table II).

TABLE II

RETENTION TIMES OF SOME COMPOUNDS

Peak No.*	Compound	Retention time (min)
1	Acetaminophen	3.0
	Sulfadiazine	3.0
	Cefuroxime	3.1
	Theobromine	3.4
	β-Hydroxyethyltheophylline	4.2
	1,7-Dimethylxanthine	4.4
2	Theophylline	4.6
	Sulfamethizole	4.9
	Sulfamethoxidiazine	5.0
3	Salicylate	6.2
	Sulfametoxazole	6.4
	Sulfafurazole	7.9
	Salicylamide	8.1
4	8-Chlorotheophylline	8.7
	Persedon	9.1
	Caffeine	10.2
	3-Isobutyl-1-methylxanthine	>15

*The drugs are numbered as the peaks in Fig. 1.

All compounds either eluted later than 20 min after injection or did not elute at all.

Table II shows the retention times of some compounds detected by the procedure. A short comparison between theophylline analyzed by HPLC and enzyme-multiplied immunoassay technique (EMIT) (according to our earlier reported procedure for the Gemsaec centrifugal fast analyzer [28]) is given in Table III.

TABLE III

COMPARISON OF THEOPHYLLINE ANALYZED BY HPLC AND EMIT

Patient code	Theophylline (μg/ml)	
	EMIT	HPLC
1	19.8	18.7
2	5.8	7.6
3	12.7	13.5
4	15.3	15.6
5	12.2	12.5
6	9.6	9.8
7	15.6	15.6
8	12.4	12.9
9	10.5	10.9
10	8.0	8.5

DISCUSSION

As seen in Table II, β-hydroxyethyl-theophylline was not resolved from theophylline and could therefore not be used as an internal standard in our procedure (β-hydroxyethyltheophylline is a commonly used internal standard for the determination of theophylline by HPLC).

In most cases a patient's medication is known and therefore unknown peaks are not likely to be found in the chromatograms. However, in situations of suicidal drug overdose, confirmation of peak identity is of prime interest since the drug "panorama" then may be unknown and complex. UV spectra of eluted peaks may help identify the drugs.

The procedure has now been used in our laboratory for half a year, primarily to determine theophylline in sera from patients undergoing theophylline therapy. Possible interference from compounds like sulfonamides and antibiotics has to be considered. We recommend others to investigate the interference from the latter groups of compounds. The chemical structures of these compounds in medical use may differ from country to country. The use of an extraction technique to recover the drugs from serum is beneficial since the extracts are relatively free from endogenous interfering compounds and suspended particles.

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