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Note

Rapid method of measuring salicylate in serum by high-performance liquid chromatography

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Measurement of the concentration of salicylate in body fluids is normally carried out in connection either with the diagnosis and management of patients suffering from salicylate intoxication, or with studies of the pharmacokinetics of acetylsalicylic acid (aspirin) and its derivatives. The colorimetric assay [1] most frequently used in the former situation, which is based on the reaction between salicylate and iron(III) ions to form a purple complex, is simple and inexpensive, but suffers interference from several compounds [2]. Many methods of determining salicylate and certain of its metabolites using gas chromatography and high-performance liquid chromatography (HPLC) have been described, mainly for use in pharmacokinetic studies (for example, see refs. 3—7) and these are specific and sensitive, but are generally not suitable for use in clinical laboratories due to the expense of the equipment required and the time taken to run single assays. The present paper describes a rapid method of measuring salicylate in serum by HPLC which was developed to provide a reference method with which the performance of new emergency salicylate assays could be compared.

EXPERIMENTAL

Sodium salicylate (99+%) was obtained from Aldrich (Gillingham, U.K.).

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Salicyluric acid, gentisic acid, acetylsalicylic acid, *o*-methoxybenzoic acid, reduced nicotinamide-adenine dinucleotide (NADH) and bovine albumin were obtained from Sigma (Poole, U.K.) and acetic acid was supplied by BDH (Dagenham, U.K.). Methanol and acetonitrile were of HPLC grade and were supplied by Rathburn Chemicals (Walkerburn, U.K.). Salicylate 1-monooxygenase was obtained from the Centre for Applied Microbiology and Research (Porton Down, U.K.).

The chromatograph consisted of a dual-piston pump (M6000A), a radial compression module (Z-module) fitted with a C_{18} μBondapak cartridge (10 cm × 8 mm; 10 μm particle size) and guard column, a spectrophotometric detector (Model 480) set to 315 nm and 0.2 absorbance units full scale (a.u.f.s.) range and a plotter/integrator (Model 720 data module) (all supplied by Millipore-Waters, Harrow, U.K.). Samples were loaded by means of an injector valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.) fitted with a 20-μl loop. The mobile phase consisted of 47.5% methanol and 5% acetic acid in water, and was filtered through a 0.45-μm membrane filter (Millipore) under reduced pressure before use. The flow-rate was 3.0 ml/min.

To 100 μl of sample or standard in a microcentrifuge tube was added 200 μl of acetonitrile containing *o*-methoxybenzoic acid (550 mg/l). The tubes were capped and vortex-mixed for 15 s each (although extraction was found to be complete after only 5 s) and centrifuged for 2 min at 10 000 g_{max}. Aliquots (100 μl) of the supernatant solution were then loaded into the injector loop and the chromatograms started by opening the loop valve. Salicylate concentrations were determined on the data module by the peak-area ratio method using *o*-methoxybenzoic acid as the internal standard. The data module was calibrated using standard solutions of sodium salicylate (4.0 mmol/l) in either normal human serum or water.

The colorimetric method was carried out essentially as described by Trinder [1].

The salicylate concentration in a limited number of serum samples was also measured enzymatically using salicylate 1-monooxygenase (EC 1.14.13.1), which catalyses the decarboxylation and hydroxylation of salicylate with concomitant oxidation of an equal amount of NADH [8]. To 1.5 ml of a reagent consisting of salicylate 1-monooxygenase (1 U/ml), NADH (0.5 mmol/l), bovine albumin (1 g/l) and tris(hydroxymethyl)methylamine (0.1 mol/l; pH adjusted to 8.5 with hydrochloric acid) in a 0.5-cm pathlength cuvette was added 75 μl of sample. The absorbance of the solution at 340 nm was monitored using an SP6-550 spectrophotometer (Pye Unicam, Cambridge, U.K.) before and for 3 min after addition of the sample, and the salicylate concentration determined from the NADH consumed, assuming the molar extinction coefficient for NADH at 340 nm to be $6.22 \cdot 10^3$ l mol^{-1} cm^{-1} [9]. Appropriate corrections for the NADH oxidase activity of the enzyme [8] and for the absorbance of the samples were applied.

RESULTS

Fig. 1 shows typical chromatograms obtained with the HPLC method. In salicylate-free serum treated with acetonitrile without *o*-methoxybenzoic acid,

Fig. 1. (a) Chromatogram obtained with serum from a subject known not to be taking salicylates; no internal standard. (b) Chromatogram obtained with a standard solution of salicylate (4.0 mmol/l). (c) Chromatogram obtained with serum from a patient suffering from salicylate intoxication. The salicylate concentration was 3.1 mmol/l. Each chromatogram was run for 5 min; the values along each peak are retention times (min). Peaks: MBA = *o*-methoxybenzoic acid, internal standard; SA = salicylic acid.

there were no detectable compounds between 1.5 and 5.0 min (Fig. 1a). With both a standard salicylate solution, and serum from a patient who had taken an overdose of acetylsalicylic acid (aspirin), *o*-methoxybenzoic acid and salicylate eluted after 2.25 and 3.18 min, respectively, and in both cases only two peaks between 1.5 and 5.0 min were observed (Fig. 1b and c). Salicylate was completely eluted within 4 min.

The response of the data module to increasing salicylate concentrations was found to be linear up to at least 5 mmol/l. When seven standard solutions with concentrations in this range were assayed singly, the regression line of peak-area ratio against concentration had a correlation coefficient of 0.99997.

The precision of the method was determined by performing twenty assays of each of two samples of normal human serum spiked with low and high concentrations of sodium salicylate. The mean concentrations ± standard deviations and coefficients of variation obtained were 0.484 ± 0.005 mmol/l, 1.03% and 3.13 ± 0.02 mmol/l, 0.64%, respectively.

The accuracy of the method was determined in several ways. Firstly, the recovery of salicylate from ten serum samples spiked to a final concentration of 2.0 mmol/l was between 95.0 and 103.5%. Secondly, samples of serum contain-

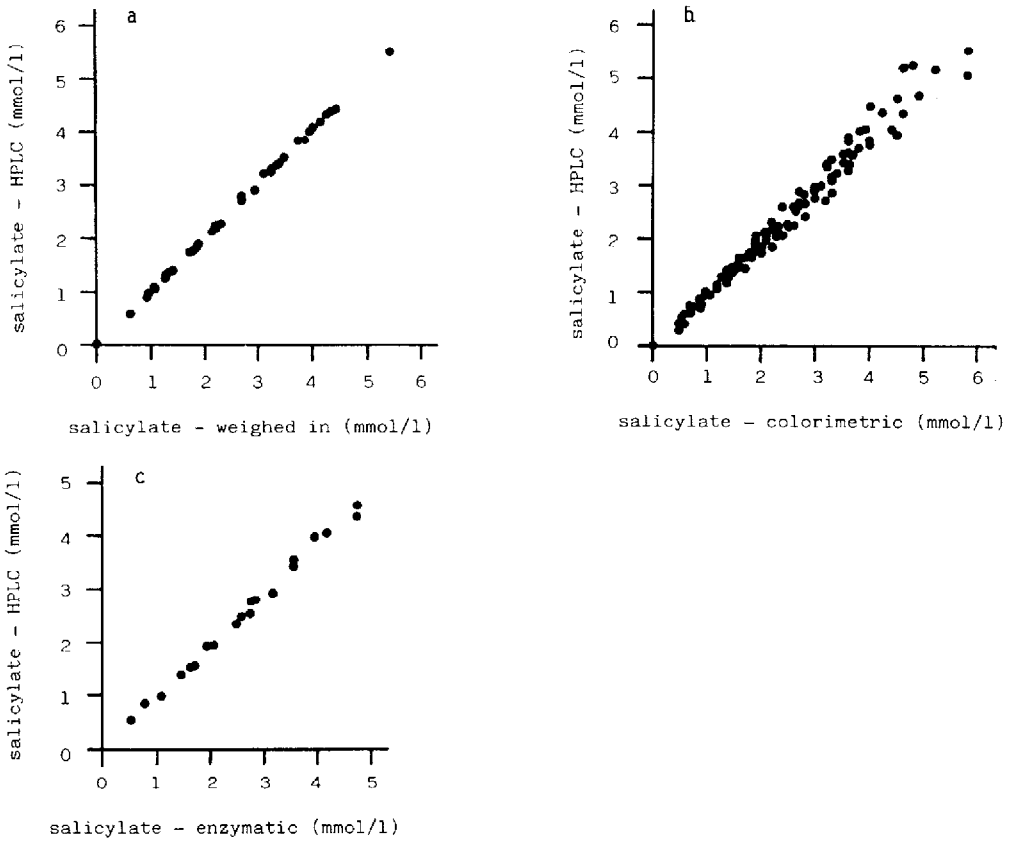

Fig. 2. Performance of the chromatographic salicylate assay. (a) Performance in an External Quality Assessment Scheme; $y = 1.006x - 0.031$, $r = 0.999$, $n = 41$. (b) Comparison of results obtained by the chromatographic and colorimetric assays on intoxicated patients' samples; $y = 0.990x - 0.051$, $r = 0.992$, $n = 142$. (c) Comparison of results obtained by the chromatographic and enzymatic assays on intoxicated patients' samples; $y = 0.971x - 0.036$, $r = 0.997$, $n = 20$. Regression parameters were calculated using the Deming procedure [10].

ing salicylate which had been issued to Clinical Chemistry laboratories as part of an External Quality Assessment Scheme were analysed and the results compared to the "weighed-in" concentrations. The results, plotted in Fig. 2a, show that there was very close agreement between the two sets of concentrations. Finally, the salicylate concentrations in serum samples from patients suffering from salicylate intoxication were measured by the HPLC method and the results compared with those obtained with the colorimetric assay [1] and the enzymatic assay described above. The results are shown in Fig. 2b and c, which show that there was good agreement between the three methods.

Interference in the assay by the compounds shown in Table I was tested in two stages. Firstly, 100 μl of a solution of each compound in salicylate-free serum was extracted with acetonitrile, and the supernatant solution chromatographed as described above, to determine which compounds gave detectable peaks under the conditions of the assay. Secondly, the effect on the

TABLE I

COMPOUNDS TESTED FOR INTERFERENCE IN THE CHROMATOGRAPHIC SALICYLATE ASSAY

Structurally related compounds and ketones (5 mmol/l)	Drugs (0.5 g/l)	Drugs (0.5 g/l)
Acetoacetate	N-Acetylcysteine	Paracetamol
o-Acetylsalicylic acid	Benzylpenicillin	Phenacetin
4-Aminosalicylate	Caffeine	Phenobarbitone
Benzoate	Carbamazepine	Phenylephrine
Gentisic acid	Diazepam	Phenytoin
4-Hydroxybenzoate	Ethoxsuximide	Procainamide
3-Hydroxybenzoate	Hexobarbitone	Salicylamide
Salicyluric acid	Imipramine	Sulfarmethoxazole
	Methocarbamol	Theobromine
	Metoclopramide	Theophylline

TABLE II

RETENTION TIMES AND EFFECTS ON THE RECOVERY OF SALICYLATE OF COMPOUNDS DETECTABLE BY THE CHROMATOGRAPHIC ASSAY

Compound	Final concentration	Retention time (min)	Recovery (%)
4-Aminosalicylate	5 mmol/l	1.68	100.5
Carbamazepine	0.5 g/l	4.40	95.3
Diazepam	0.5 g/l	8.81	N.T.*
Gentisic acid	5 mmol/l	1.81	102.6
Metoclopramide	0.5 g/l	2.55	150.8
Procainamide	0.5 g/l	1.58	97.9
Salicylamide	0.5 g/l	1.96	101.0
Salicyluric acid	5 mmol/l	1.95	95.8
Sulfamethoxazole	0.5 g/l	1.71	96.9

*N.T. = Not tested.

recovery of salicylate of the detectable compounds which had retention times between 1.5 and 5.0 min was tested by mixing solutions of each compound at twice the concentrations shown in Table I with equal volumes of 4.0 mmol/l salicylate in serum, and measuring the apparent salicylate concentration in the usual manner. The results, presented in Table II, show that only metoclopramide had a significant effect on the recovery of salicylate. Salicyluric acid and gentisic acid, which are metabolites of salicylate, and the structurally related compounds 4-aminosalicylate and salicylamide had little effect.

The recovery of salicylate from samples containing high concentrations of haemoglobin (500 mg/dl) and bilirubin (600 μmol/l) was measured; no effect was noted.

DISCUSSION

This paper has described a rapid, accurate and precise method of determining salicylate concentrations in serum using HPLC. The speed of the method is attributable to two factors. Firstly, the extraction of salicylate is complete after only 5 s mixing with acetonitrile; this is clearly a considerable advantage over those methods which involve solvent evaporation [6] or back-extraction [7], especially when large numbers of samples are to be analysed. Acetonitrile has been used successfully in the extraction protocol for a variety of analytes using HPLC techniques [11]. This rapid extraction was not gained at the cost of interference from endogenous compounds in serum or from salicylate metabolites, and there was no indication from recovery experiments that the extraction was incomplete. Secondly, under the conditions of the assay, salicylic acid was eluted from the column after less than 4 min. No attempt was made to resolve the salicylate metabolites salicyluric acid and gentisic acid, which were eluted within 2 min, since they do not contribute to the pathogenesis of salicylate intoxication [12]. Quantification of these compounds as well as salicylate, if desired, would necessitate a longer analysis time.

The sensitivity of the method to salicylate was high; the peak given by a 5 mmol/l solution was accommodated by the 0.2 a.u.f.s. range. Since the detector readily operates at attenuations of 0.005 a.u.f.s. and lower, much lower concentrations of salicylate could be determined without altering the sample volume required, although the precision of the assay would be expected to decrease.

The use of a cartridge-type column and the radial compression system has resulted in a robust assay, in that over 500 samples were analysed using only one cartridge over eight months with no significant variation in the retention times of the internal standard and salicylic acid. Furthermore, the column was always quick to equilibrate, and gave an exceedingly stable baseline.

The chromatographic salicylate assay described in this paper showed excellent accuracy when samples from an External Quality Assessment Scheme were assayed, and results on samples from intoxicated patients agreed very closely with results from other assays based on different analytical principles. Furthermore, it is highly specific for salicylate; none of the salicylate metabolites or structurally related compounds tested had any significant effect on the recovery of salicylate, and of the range of drugs tested, only metoclopramide interfered significantly. These characteristics, and the speed with which individual samples can be processed once the chromatograph is running, render the procedure particularly suitable for use as a reference method when assessing the performance of new salicylate assays.

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REFERENCES

1 P. Trinder, Biochem. J., 57 (1954) 301.
2 C.S. Frings, CRC Crit. Rev. Clin. Lab. Sci., 4 (1974) 357.
3 Y.K. Tam, D.S.L. Au and F.S. Abbott, J. Chromatogr., 174 (1979) 239.
4 B.E. Cham, D. Johns, F. Bochner, D.M. Imhoff and M. Rowland, Clin. Chem., 25 (1979) 1420.
5 B. Kinberger and A. Holmén, J. Chromatogr., 229 (1982) 492.
6 J.N. Buskin, R.A. Upton and R.L. Williams, Clin. Chem., 28 (1982) 1200.
7 R.J. O'Kruk, M.A. Adams and R.B. Philp, J. Chromatogr., 310 (1984) 343.
8 R.H. White-Stevens and H. Kamin, J. Biol. Chem., 247 (1972) 2358.
9 B.L. Horecker and A. Kornberg, J. Biol. Chem., 175 (1948) 385.
10 P.J. Cornbleet and N. Gochman, Clin. Chem., 25 (1979) 432.
11 P.M. Kabra, B.E. Stafford, D.M. McDonald and L.J. Marton, in P.M. Kabra and L.J. Marton (Editors), Liquid Chromatography in Clinical Analysis, Humana Press, Clifton, NJ, 1981, p. 117.
12 G. Levy, Pediatrics, 62 (1978) 867.