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Note

Assay of underivatized salicylamide in plasma, saliva and urine

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Several methods have been described for the determination of salicylamide (SAM) in plasma and urine. These comprise UV spectrophotometric [1—5], spectrofluorimetric [6] and gas—liquid chromatographic (GLC) methods [7, 8]. The UV spectrophotometric methods exhibit lack of sensitivity (detection limit about 10 μg SAM per ml sample). The spectrofluorimetric method is more sensitive (0.5 μg SAM per ml sample), but lacks specificity and blank values have to be subtracted [6]. The published GLC methods either require the formation of trimethylsilyl derivatives [7], or lack sensitivity [8].

We developed a rapid and sensitive method for the determination of underivatized SAM in human plasma and saliva and rat whole blood, using gas chromatography with nitrogen selective detection and solid injection system.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 5750 gas chromatograph was used with an alkali flame ionization detector (nitrogen detector, HP 15151A, rubidium bromide crystal). Temperatures: injection port 280°; column 200°; detector 380°. A silanized borosilicate column (1.20 m × 0.8 mm I.D.), filled with 2% OV-225 + 1% OV-17 on Gas-Chrom Q, with a particle size of 200—220 μm, was used. Gas flow-rates: hydrogen 20—30 ml/min; air 180—200 ml/min; carrier (He) through the column 10 ml/min; auxiliary gas (He) was added to obtain a total carrier flow-rate of ± 60 ml/min. A solid injection system was used which has been described earlier for the determination of underivatized antiepileptic drugs [9] and underivatized nitrazepam and clonazepam [10].

Materials

The following materials were used: freshly distilled diethyl ether (Baker, Deventer, The Netherlands), kept over basic Al_2O_3 (Woelm) activity grade I; salicylamide (Reinst; Merck, Darmstadt, G.F.R.); N-methylhexobarbital (internal standard IS; synthesized by Drs. N.P.E. Vermeulen, by methylation of hexobarbital with diazomethane); ethanol (AR; Baker); glucuronidase and sulfatase (Limpet Acetone Powder, Patella Vulgata, crude, Type 1; Sigma, St. Louis, Mo., U.S.A.).

Extraction procedures

Extraction of SAM from human plasma and saliva and rat whole blood. To 1.0 ml plasma or saliva in a centrifuge tube, were added 25 μl ethanol containing 2.5 μg N-methylhexobarbital as internal standard and 1.0 ml acetate buffer (0.1 *M*, pH 5.0). After homogenization the mixture was extracted twice with 5 ml diethyl ether on a Cenco whirlmixer for 10 sec. The upper organic layer was removed each time with a pasteur pipette and transferred to a conical evaporation tube. The solvent was evaporated to dryness at 50—60° in a flow of dry nitrogen. The residue was dissolved in 0.1 ml of absolute ethanol and 2 μl of this solution were brought on the needle of the solid injection system. After evaporation of the solvent the needle was injected into the gas chromatograph. Blood samples (0.1 ml) of rats were analyzed in the same way, except that the extraction was carried out twice with 2 ml of diethyl ether.

Extraction of SAM from urine after hydrolysis. To 50 μl urine in a centrifuge tube of 10 ml, were added 2 ml acetate buffer (0.1 *M*, pH 4.9) containing 25 mg enzyme (9250 Fishman Units glucuronidase and 100 Enzyme Units sulfatase). After homogenization the mixture was incubated on a shaking waterbath for 3 h at 37°. After incubation, 25 μl ethanol were added containing 2.5 μg hexobarbital and the mixture was homogenized. Extraction was carried out in the same way as described for the extraction of SAM from plasma, except that 2 ml of diethyl ether were used.

Preparation of calibration curves

The concentrations of SAM in human plasma and saliva, whole blood (rat) and urine were calculated with the aid of calibration curves prepared by adding known amounts of SAM to 1.0 ml plasma or 50 μl urine. The samples were analyzed by the same procedures as described above and the ratios of the peak areas of SAM to internal standard were plotted against known concentrations of SAM. The same procedure was followed for estimation of the extraction-yield of SAM at various concentrations, except that now N-methylhexobarbital was used as an external standard. The ratios found were compared to the ratios of standard amounts of the drugs.

RESULTS AND DISCUSSION

Fig. 1 shows the gas chromatograms of the extracts from human plasma and saliva samples. There is no interference from endogenous substances according to the blank, the retention time is short and the detection limits are

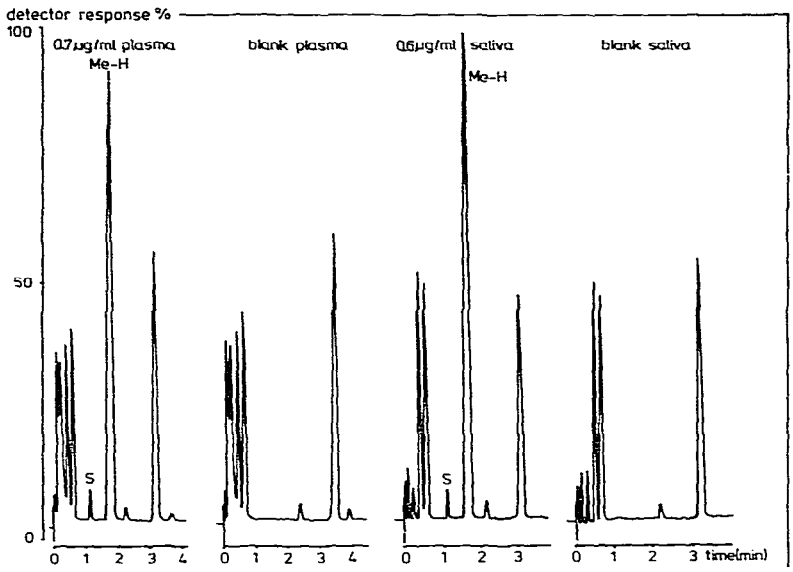

Fig. 1. Gas chromatograms of extracts of 1 ml plasma and saliva, obtained from a volunteer after 2½ and 1½ hours, respectively, and before (blank plasma and blank saliva) administration of 1500 mg salicylamide orally.
S = Salicylamide, Me-H = N-methylhexobarbital (IS, 2.5 µg/ml plasma or saliva). Salicylamide concentration: in plasma, 0.7 µg/ml; in saliva, 0.6 µg/ml.

about 0.25 µg SAM per ml plasma or saliva, or 0.25 µg SAM per 0.1 ml rat whole blood. Identification of the compounds eluting from the gas chromatograph was carried out by means of combined gas chromatography—mass spectrometry (LKB-2091 with PDP-11 computer system).

Metabolites [3] of SAM (SAM sulfate, SAM glucuronide, gentisamide and gentisamide glucuronide) do not interfere, primarily because these compounds are not co-extracted using the present extraction procedure. On the other hand the conjugates can be readily determined by the present assay, after hydrolysis (glucuronidase and sulfatase incubation) to the parent drug.

Linearity exists between detector response (expressed as the ratio of the peak area SAM:peak area N-methylhexobarbital) and the concentration of SAM from 1.0 to 25.0 µg/ml human plasma and saliva.

The mean of three plasma and saliva calibration curves obtained on different occasions showed, for each point in the concentration range from 1.0 to 25.0 µg SAM/ml, a standard deviation (S.D.) which was smaller than 5%. In spite of the short extraction time (10 sec) extraction yields of SAM from whole blood (rat) and human plasma and saliva are relatively high: 97%, 83% and 83%, respectively, and are constant over the concentration range 1–25 µg SAM per ml. Calibration curves for the extraction of SAM from urine (human and rat) after enzymatic hydrolysis were linear. In the concentration range of 1—5 µg SAM per 50 ml urine and 5—100 µg SAM per 50 µl urine different calibration curves were used, because of a bend in the calibration curve. The enzymatic hydrolysis was performed within 3 h instead of 16 h [3]. The calibration curve obtained following either hydrolysis time was repro-

ducible, with the difference that after hydrolysis for 3 h larger amounts of SAM were determined probably due to the fact that less was broken down in the shorter period. This was also noted by Levy and Matsuzawa [3], and was checked by adding known amounts of SAM to blank urine and performing the hydrolyses over 3 and 16 h, respectively. The results showed that the calibration curve after the 3-h hydrolysis was significantly higher than after 16 h. Further experiments showed that 25 mg enzyme was sufficient even for hydrolysis of large quantities of SAM-metabolites (equivalent to approximately 100 μg SAM per 50 μl urine) to SAM within 3 h.

Larger quantities of enzyme and longer hydrolysis times did not show any increase in SAM recovery. The recovery of SAM from urine (human and rat) after incubation with glucuronidase and sulfatase was 92% (S.D. < 5.0%; n = 3) in the concentration range of 5 –100 μg per 50 μl urine. In the urine assay of SAM, hexobarbital was used as IS because of an interfering peak when N-methylhexobarbital was used. Attempts were made to analyse gentisamide sulfate and gentisamide glucuronide in urine as gentisamide (a minor metabolite [3]), however, this compound decomposed during the enzymatic hydrolysis and could therefore not be accurately determined.

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