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Note

Determination of metformin in biological fluids by derivatization followed by high-performance liquid chromatography

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Metformin (1,1-dimethylbiguanide) is used clinically as an oral hypoglycaemic agent. It is a member of the group of biguanide compounds, all of which present problems in analysis, particularly at pharmacological dose levels. Methods described for the determination of this group of pharmaceuticals include a colorimetric assay[1], fluorescence[2], fluorescence quenching[3], pyrolysis gas chromatography[4] and ion-pair extraction[5].

All chromatographic methods are complicated by the polar nature of the biguanides. One way of surmounting this problem is to convert them into compounds more amenable to chromatographic analysis. A survey of the chemical literature reveals that biguanides react readily with acid anhydrides and acid chlorides[6] to form *s*-triazine derivatives. Matin *et al.*[7] utilized this reaction to produce 4-monochlorodifluoromethyl-*s*-triazine derivatives which could be analysed by gas chromatography using electron capture detection, or 4-trifluoromethyl-*s*-triazine derivatives which were analysed by gas chromatography with chemical ionization mass spectrometry[8]. We report the derivatization of metformin to produce a compound with good UV-absorbing characteristics which can be analysed by high-performance liquid chromatography (HPLC) and describe an analytical procedure suitable for the determination of metformin in urine.

EXPERIMENTAL

Preparation of 2-amino-6-dimethylamino-4-(4'-nitrophenyl)-s-triazine

Metformin hydrochloride (100 mg) in acetonitrile (1.0 ml) and sodium hydroxide (20% aqueous, 1.0 ml) was treated with *p*-nitrobenzoyl chloride (100 mg) and the reaction allowed to proceed at room temperature for 6 h. Water (10 ml) was added and the reaction product obtained by filtration. Re-crystallisation from methanol–water (3:2) afforded the substituted triazine derivative, melting point 258–260° (148 mg, 94% yield).

Derivatization of metformin for HPLC analysis

Urine samples requiring analysis (2 ml) were treated with aqueous sodium hydroxide solution (1.0 ml, 20%) and saturated with solid sodium chloride. Aceto-

nitrile (1.0 ml) and *p*-nitrobenzoyl chloride (10 mg approx.) were added to the urine sample and the mixture was maintained at room temperature for 1 h. A further 10 mg of *p*-nitrobenzoyl chloride were added and after 1 h the 10-μl aliquots of the acetonitrile solution were taken for analysis.

HPLC analysis

Ten microlitres of the above solution were subjected to HPLC analysis. Analyses were performed at ambient temperatures on a 3 ft. $\times$ 0.085 in. I.D. stainless-steel column packed with Bondapak™ phenyl/Corasil (particle size 37–50 μm; Waters Assoc., Milford, Mass., U.S.A.). The eluent flow-rate was 1 ml/min (pressure 800 p.s.i.) generated by a Model 6000M solvent delivery system (Waters Assoc.). The eluent was 40% methanol (Analar grade) in water. An UV spectrophotometer (Cecil Instruments CE 272 with modified flow cell) monitoring at 280 nm was used as detector. All calibrations and analyses were carried out with 10-μl injection size.

DISCUSSION AND RESULTS

p-Nitrobenzoyl chloride has attracted interest as an HPLC derivatization agent; it is used to form nitro esters by reaction with free hydroxyl groups, thus allowing the UV detection of compounds that do not of themselves possess suitable UV-absorbing properties[9]. This reagent will also serve to convert biguanides to *s*-triazine derivatives. The reaction with metformin is shown in Fig. 1. The metformin derivative was prepared by a modification of the method of Shapiro *et al.*[6] and its structure confirmed by standard methods. The mass spectrum and proposed fragmentation pathway is shown in Fig. 2.

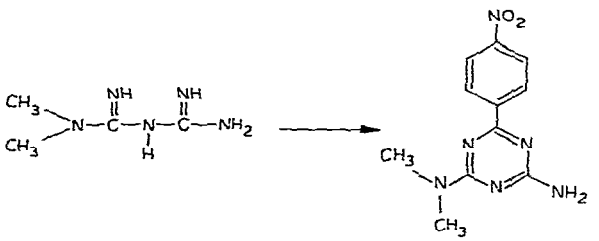

Fig. 1. Metformin and its *s*-triazine derivative.

HPLC on Bondapak phenyl/Corasil with 40% methanol in water as eluent and UV detection at 280 nm produced the chromatogram shown in Fig. 3a. Samples of urine containing various amounts of metformin hydrochloride were treated with 20% aqueous sodium hydroxide, the resulting solution saturated with sodium chloride[7] and acetonitrile added. Provided the solution is saturated with sodium chloride, the acetonitrile is immiscible and remains as the top layer. Excess *p*-nitrobenzoyl chloride was added as solid and the reaction allowed to proceed at room temperature. HPLC of the organic phase showed the production of a compound that co-eluted with the previously prepared derivative (Fig. 3b) and its identity was further confirmed by collecting the eluted compound and subjecting it to mass spectroscopy where it gave

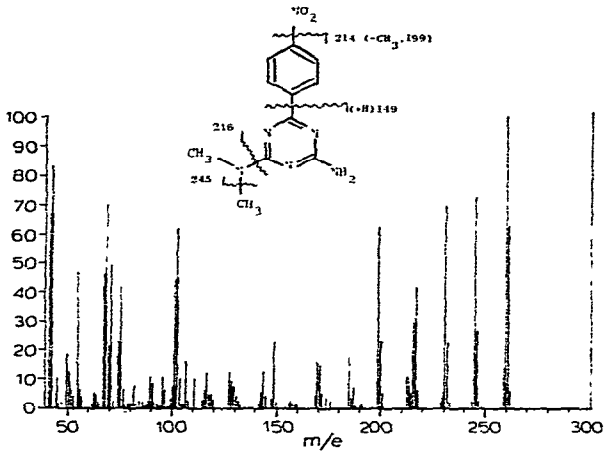

Fig. 2. Mass spectrum and proposed fragmentation pathway of the *s*-triazine derivative of metformin.

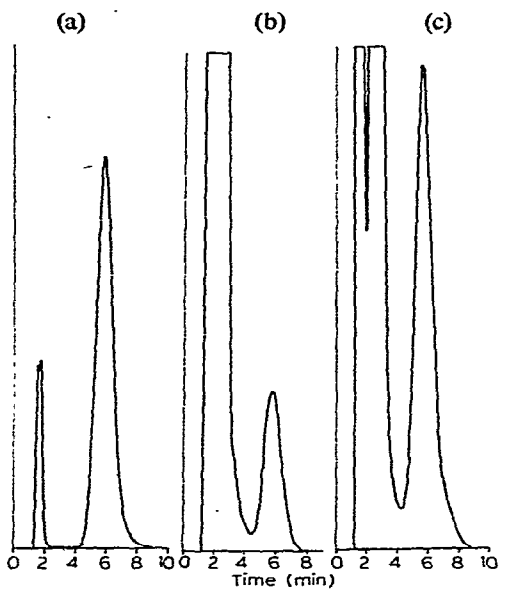

Fig. 3. HPLC of metformin derivative. (a) Synthesized triazine; (b) after derivatization from urine sample; (c) *p*-nitrobenzoic acid (unretained), *p*-nitrobenzoyl chloride and triazine derivative.

a spectrum identical to that shown in Fig. 2. By HPLC the reaction was shown to have ceased within 2 h, however a calibration curve of concentration *versus* response was not linear (line a in Fig. 4). In this derivarization procedure there are two competing reactions, the conversion of metformin to the *s*-triazine derivative and the base hydrolysis of *p*-nitrobenzoyl chloride to produce sodium nitrobenzoate, which remains in the aqueous phase. With the higher concentrations of metformin it appeared that, although a theoretical excess of reagent was added, the reaction rates of the two reactions were such that unreacted metformin was still present. This was confirmed by adding more *p*-nitrobenzoyl chloride to the supposedly complete reactions. Re-analy-

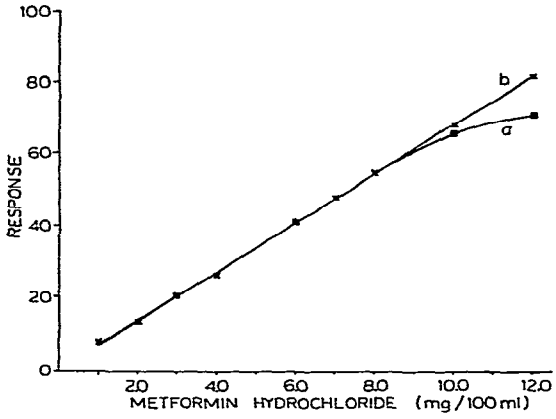

Fig. 4. Calibration curves for metformin hydrochloride in urine. See text for explanation of lines a and b.

sis after 1 h showed that those samples that deviated from a straight-line calibration increased in response. Modification of the derivatization procedure involving a second addition of *p*-nitrobenzoyl chloride after 1 h produced a straight calibration line (line b in Fig. 4). The HPLC characteristics of *p*-nitrobenzoyl chloride and *p*-nitrobenzoic acid are shown in Fig. 3c and it can be seen that *p*-nitrobenzoic acid (which should not be present in the acetonitrile phase in any case) is unretained and *p*-nitrobenzoyl chloride has a retention time between the acid and the triazine derivative. Where *p*-nitrobenzoyl chloride is present in great excess it may interfere with the accurate determination of the derivative response but by the end of the stipulated reaction time no *p*-nitrobenzoyl chloride is detected.

This method allows the estimation of metformin hydrochloride in urine at concentrations of 1.0 mg/100 ml of urine. Modification of the extraction procedure involving an increase in the amount of urine and reduction of the amount of acetonitrile allowed estimation of concentrations as low as 0.02 mg/100 ml urine.

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