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Methotrexate: Existence of different types of solid

Hak-Kim Chan and Igor Gonda

Department of Pharmacy, University of Sydney, Sydney, NSW 2006 (Australia)

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Summary

Methotrexate (MTX) was found to exist as four types of solid materials. Two of these appear to be different habits of the stable pseudopolymorph (hydrate), a tetragonal crystalline form containing water of crystallization. The originally supplied commercial powder has a lower degree of crystallinity than the above solids and also exhibits differences in the infrared spectra, X-ray diffraction pattern, differential thermal analysis, differential scanning calorimetry and thermogravimetry. It also gives higher aqueous concentrations of dissolved MTX but on prolonged standing, it reverts to the more stable hydrate. The most soluble form of MTX is an amorphous powder which does not revert to a more stable crystalline state even when kept in contact with water for about 100 h.

Introduction

Methotrexate (MTX) is a well known antitumour agent which acts by inhibition of the ubiquitous enzyme dihydrofolate reductase. In order to improve the selective toxicity of this drug, we have attempted to develop solid forms of MTX suitable for direct pulmonary delivery for treatment of lung cancer in the form of a dry powder aerosol (Chan et al., 1986; Hambley et al., 1986; Chan, 1988; Chan and Gonda, 1989). Our initial effort was hampered by the lack of crystallinity of the commercially available MTX powder. In an attempt to prepare, instead, a crystalline complex of MTX with thymidine, we have serendipitously obtained well-developed tetragonal crystals of pure MTX (Chan, 1988; Chan and Gonda, 1989) which led the way to the preparation of such crystals from a variety of systems including pure water. These particles were subjected to single crystal X-ray diffraction analysis, and the crystal and molecular (conformation) structure were thus established (Chan et al., 1986; Hambley et al., 1986). During those investigations, a number of apparently different solid forms of MTX was observed. It is the purpose of this communication to report on the results of the tests to identify these solid forms.

Correspondence: I. Gonda, Department of Pharmacy, University of Sydney, Sydney, NSW 2006, Australia.

Materials and Methods

All solvents were of analytical grade. Methotrexate (American Cyanamid Co., Pearl River, U.S.A.) was analyzed either as the original powder

(O) from the manufacturer, or as one of the following materials:

Anhedral particles (A): 1 g MTX was dissolved in 800 ml hot (boiling) methanol and the solution was allowed to cool to room temperature.

Tetragonal crystals (T): 1 g MTX was dissolved in 500 ml hot (90–100°C) double-distilled water and the solution was allowed to cool and form crystals spontaneously.

'Spherical' particles (S): 0.5 g MTX and 1 g β-cyclodextrin (Celdex, Nihon Shokuhin Kako Co. Ltd, Japan) were dissolved in 150 ml water at 90–100 °C, then allowed to cool for spontaneous nucleation and crystallization. A number of other 'additives' was found to promote formation of apparently spherical particles of MTX. β-cyclodextrin was selected for the subsequent tests because this compound could be readily distinguished from MTX in the ^{1}H-NMR spectrum so that a complex formation with MTX would be easily detected.

All the samples were kept over silica gel in a desiccator before use.

Characterization of the solids

Polarizing microscopy

The MTX powder was observed in an immersion oil ($n = 1.53$) using a petrological polarizing microscope (Leitz, Germany).

Scanning electron microscopy (SEM)

MTX powder samples were mounted on an EM sample stub by double-sided sticky tape. After platinum coating, the samples were viewed under a scanning electron microscope (JEM 35C, Jeol, Japan) using an electron beam voltage of 15 kV and a back-scattered electron detector.

Transmission electron microscopy (TEM)

This work was carried out by Dr David Cockayne of the Electron Microscope Unit, University of Sydney. MTX powder was directly adhered onto the surface of an EM grid which was then observed under a transmission electron microscope (Model 300, Philips, The Netherlands). TEM provided a complementary study to X-ray diffraction of the crystallinity of the powder by observing the diffraction pattern of the electron beam.

Hot-stage microscopy (HSM) and melting point measurement

Melting point (m.p.) was measured by a melting point apparatus (Townson and Mercer Ltd., U.K.) using a heating rate of 4°C/min as recommended for substances which decompose on melting (Kuhnert-Brandstatter, 1966). For the thermomicroscopic investigation, a polarizing microscope (Reichert, Wien, Austria) fitted with a Kofler hot stage (Reichert, Wien, Austria) was used. The samples were heated at rates of 5–15 °C/min and thermal-physical changes were examined. This procedure was repeated using suspensions of the samples in silicone oil (200 Fluid/350 cs, Dow Corning, Ajax, Sydney, Australia) for study of desolvation (Cheronis, 1954; Kuhnert-Brandstatter and Grimm, 1968).

UV absorption spectroscopy

UV absorption spectra were recorded using a spectrophotometer (Lambda 5 UV/VIS, Perkin-Elmer, U.S.A.) with instrumental settings: slit, 2 nm; scan speed, 60 nm/min; response, 0.5 s; peak threshold, 0.02 A. A pair of matched 1 cm quartz cuvettes was used. The solvent was 0.1 N NaOH.

IR spectroscopy

IR spectra were determined in an infrared spectrophotometer (double-beam, model 297, Perkin-Elmer, U.S.A.) using the nujol method.

Mass spectrometry

The method of mass spectrometry used by Cheung et al. (1985) for the characterization of analogues of MTX was employed. Chemical ionization (CI) mass spectra were recorded on a quadrupole mass spectrometer (Model 3200, Finnigan, U.S.A.) on-line to a data system (Model 6100, Finnigan, U.S.A.). Anhydrous ammonia (99.9%) was used as reactant gas at a pressure of 1 Torr. The source temperature was 90 °C. The ammonia CI spectra were collected using a solids insertion probe for thermal desorption from a platinum wire (Cheung et al., 1985). Samples of 1–5 μg of MTX were used.

Thin-layer chromatography (TLC)

The method of assay of MTX in the USP XIX edition (USP, 1975) was used. MTX sample solutions were prepared and then applied onto the TLC plate. The chromatography system was as follows: TLC plate: cellulose-coated glass plate (Merck, length 12 cm, Germany). The developing solvent was a solution of citric acid monohydrate (10 g/200 ml) adjusted to pH 8 with NH_4OH. 10 volumes of this solution were shaken with 1 volume of amyl alcohol and allowed to stand for 30 min, after which the lower phase was used. The bands were visualized with a UV lamp (both short- and long-wavelength light). After development, drying and visualization, both the fluorescent and non-fluorescent (yellow colour) bands were scraped separately into centrifuge tubes containing 0.1 N NaOH. After shaking and centrifuging, the supernatant MTX solutions were diluted and analyzed by UV spectroscopy.

High performance liquid chromatography (HPLC)

The HPLC was carried out in an isocratic system (model 330, Altex, U.S.A.). 25 μl samples were injected using a mixture of 10% acetonitrile and 90% citric acid buffer, pH 6, as the mobile phase ran at 1 ml/min. The column was 10 cm long, and had 5 μm beads and C_{18} stationary phase. MTX was detected at 303 nm (Jasco UVIDEC - 100 - III, Japan Spectroscopic Co., Japan).

Optical rotation

Since the MTX molecule has a chiral centre (α-carbon in the glutamate moiety) and is therefore optically active, it is possible that during crystallization in hot water, racemic modification might have resulted due to thermal racemization or ion formation (Eliel, 1962). In order to check this, optical rotation of MTX in 0.1 N NaOH was measured using a polarimeter (Hilger and Watts, London, U.K.) with a 2 dm^3 sample tube and a sodium lamp as the light source.

^{1}H-NMR spectroscopy

NMR spectra were obtained in a Fourier transform instrument (Model FX 9Q, Jeol, Japan) operating at 90 MHz with deuterium lock signal for tuning, 8 K data points and spectral width 896 Hz. Tetramethylsilane (TMS) and perdeuterated dimethylsulphoxide (d_6-DMSO) were used as the internal standard and solvent, respectively.

X-ray powder diffraction

The diffraction patterns were recorded in a diffractometer (Miniflex CN 2005, Rigaku, Japan) with a Cu target, X-ray tube voltage 30 kV and scanning speed of 2°/min at 50 Hz.

Thermal analysis

A combined DTA-TG analysis was performed (Thermoflex 8075, Rigaku, Japan) by heating the samples from room temperature to approx. 250°C at 10°C/min in air. Sensitivity was ± 100 μV and an empty sample pan was used as the reference standard. DTA was also repeated under a nitrogen atmosphere. Differential scanning calorimetry was carried out on the samples at 10°C/min under a nitrogen atmosphere (Model 1090, Du Pont, U.S.A.). Also, cyclic runs by cooling and reheating the samples were carried out on a combined DTA-TG thermal analyzer (Thermoflex, CN 8078B2, Rigaku, Tokyo, Japan) using a sensitivity of ± 25 μV with ignited Al_2O_3 as reference at a heating rate of 10°C/min under N_2 flow of 30 cm^3/min. 10.0 mg samples were used except for sample A: because of its bulky volume, 7.0 mg was employed.

Moisture content determination

The total water content of the samples was determined by a Karl Fischer titration instrument (Model 392, Fisher Scientific Co., Pittsburgh, U.S.A.) using a sample size of 200 mg per titration in the methanol-pyridine solvent system.

Solubility measurement

The solubility characteristics of the four representative samples O, A, T and S were studied as follows: 100 mg of the sample were put into a glass tube (i.d. 60 mm) containing 200 ml double-distilled water (pH 6.2 ± 0.3) which was kept in a thermostatically controlled water bath at 20°C (YSI proportional temperature controller, model 72, Yellow Springs Instrument Co., OH, U.S.A.). The solutions were stirred continuously by a mag-

netic stirrer (Dynamax, Ainsworth Consolidated Industries Ltd, Sydney, Australia). At fixed times, 10 ml portions were removed and filtered by a membrane filter (0.2 μm, Metrical, Gelman Sciences, Ann Arbor, MI, U.S.A.) fitted to a syringe (the filtration equipment was equilibrated at the bath temperature). Preliminary experiments showed that in order to minimize surface adsorption onto the filter of MTX to less than 1%, the first 5 ml portion of filtrate had to be discarded so as to saturate the filter. The subsequent filtrate was diluted appropriately with 0.1 N NaOH and the MTX concentrations were determined spectrophotometrically at 303 nm (635 UV-Vis spectrophotometer, Varian Techtron, Melbourne, Australia). The calibration line of UV absorbance against concentration of MTX in 0.1 N NaOH was found to be: [absorbance = -3.5170×10^{-3} + 0.09496 × concentration (μg/ml), $r^2 = 0.9999$, $n = 8$] from which the solubilities were calculated. Since MTX is photosensitive, the whole experiment was carried out in darkness (except during sampling and absorbance determination which were performed under subdued light) to minimize possible decomposition of MTX. The solubility measurements were terminated after more than 48 h. Each of the samples was transferred to a porcelain dish with the supernatant discarded and then dried in a desiccator at room temperature in darkness. After drying, all the samples were analyzed by X-ray powder diffraction to detect any polymorphic transformation during stirring in water.

Results and Discussion

Our initial effort was to compare the original powder O with the crystals T. The original MTX powder (O) appears to be a finely divided solid with no strong crystal features when examined by optical microscopy. SEM suggests the presence of aggregates of very small particles (Fig. 1). Similarly, TEM fails to give any indication of crystallinity. Polarizing microscopy, however, reveals an anisotropic structure suggesting that the solid could be neither amorphous, nor belonging to a cubic crystal symmetry as both such solids would be isotropic and would show extinction between cross-polars.

The powder X-ray diffraction pattern of O shows definite, but low, level of crystallinity (Fig. 2). Crystallization of MTX from aqueous solutions containing high concentrations of additives such as ornithine also produces solids similar in their appearance and X-ray powder diffraction to O (Chan, 1988).

As mentioned in the Introduction, after the first crop of tetragonal MTX crystals (T) was obtained in the presence of thymidine, similar crystals were subsequently formed under a variety of conditions (even under conditions which previously only yielded apparently amorphous solids). These crystals have a slightly higher m.p. (196–210°C) than O (193–199°C) but the wide ranges and the accompanying decomposition preclude any definite conclusions.

The UV spectra in 0.1 N NaOH of O and T are identical with the characteristic maxima at 371.9, 303.1 and 258.7 nm (Chamberlin et al., 1976). The optical rotations $[\alpha]_D^{19}$ for O and T are 18 ± 2 and 19 ± 3 which indicate no racemization during the recrystallization and compare well with the literature value of 21.4 ± 0.6 at 21°C (Chamberlin et al., 1976). The mass spectra of O and T are identical (Fig. 3).

On TLC plates, O and T show a fluorescent impurity band at very low R_f values, in addition to the MTX band at $R_f = 0.4$. The polar impurities are in fact described in USP XIX (USP, 1975) as 'Methotrexate fluorescent bands' although, as mentioned previously by Zaharko and Dedrick (1984), MTX itself is not fluorescent. The original material O also contains an additional impurity band at $R_f = 0.2$. O and T show very similar HPLC elution profiles, with a major peak at a retention time of about 6.5 min.

^{1}H-NMR spectra of all four materials are identical except for a shift of peaks due to the exchangeable protons in some functional groups (Fig. 4); this evidence is important in particular to exclude the formation of a complex between MTX and β-cyclodextrin; the tetragonal crystals obtained originally by crystallization of MTX from water in the presence of thymidine also showed identical ^{1}H-NMR spectra, thus providing evi-

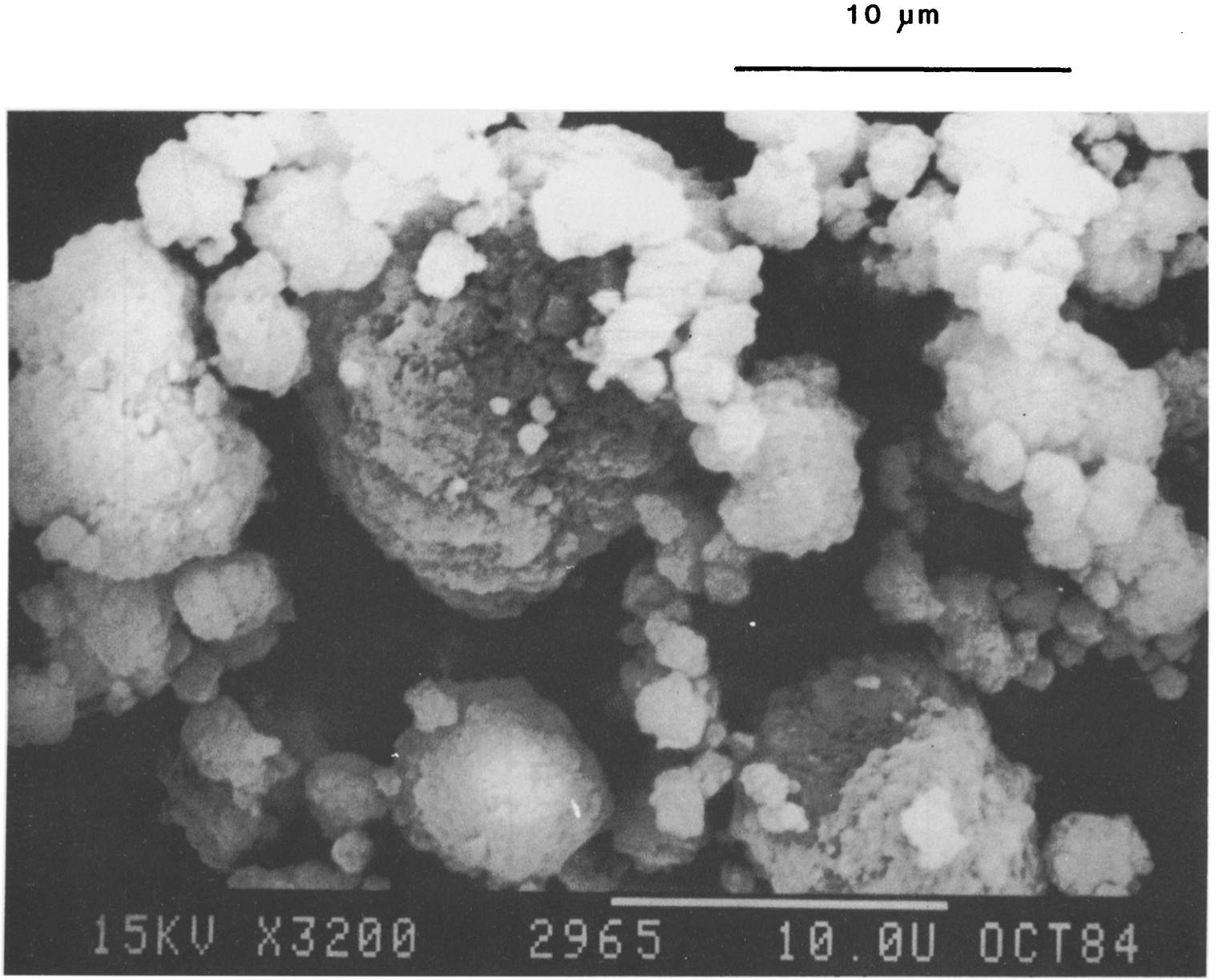

Fig. 1. Scanning electron micrograph of the original powder (O) of methotrexate.

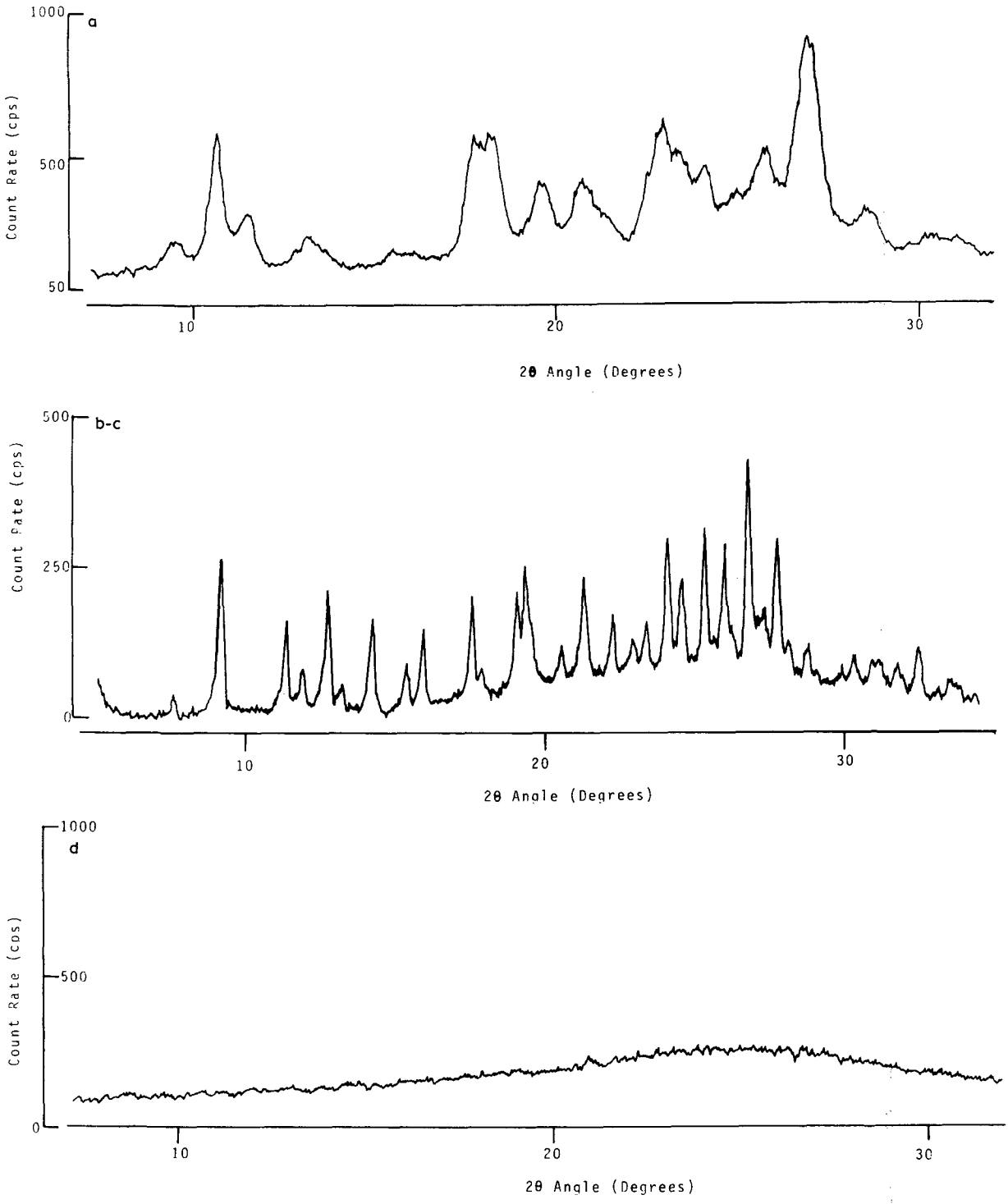

Fig. 2. X-ray powder diffraction patterns of methotrexate: (a) The original sample (O); (b) tetragonal crystals (T); (c) spherical particles (S); (d) amorphous solid (A); (e) the original sample after 100 h in a saturated aqueous solution.

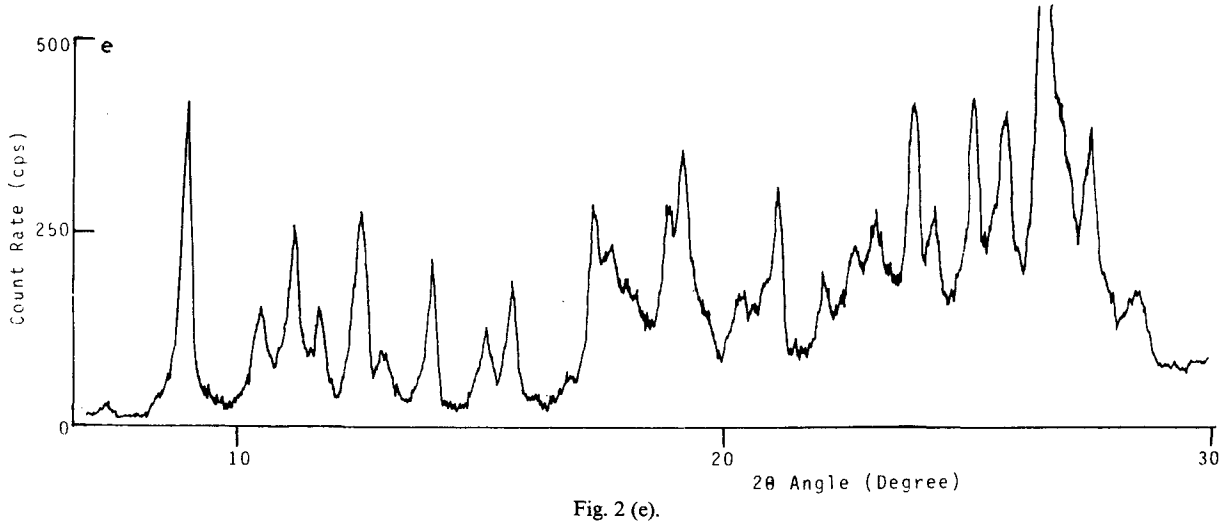

Fig. 2 (e).

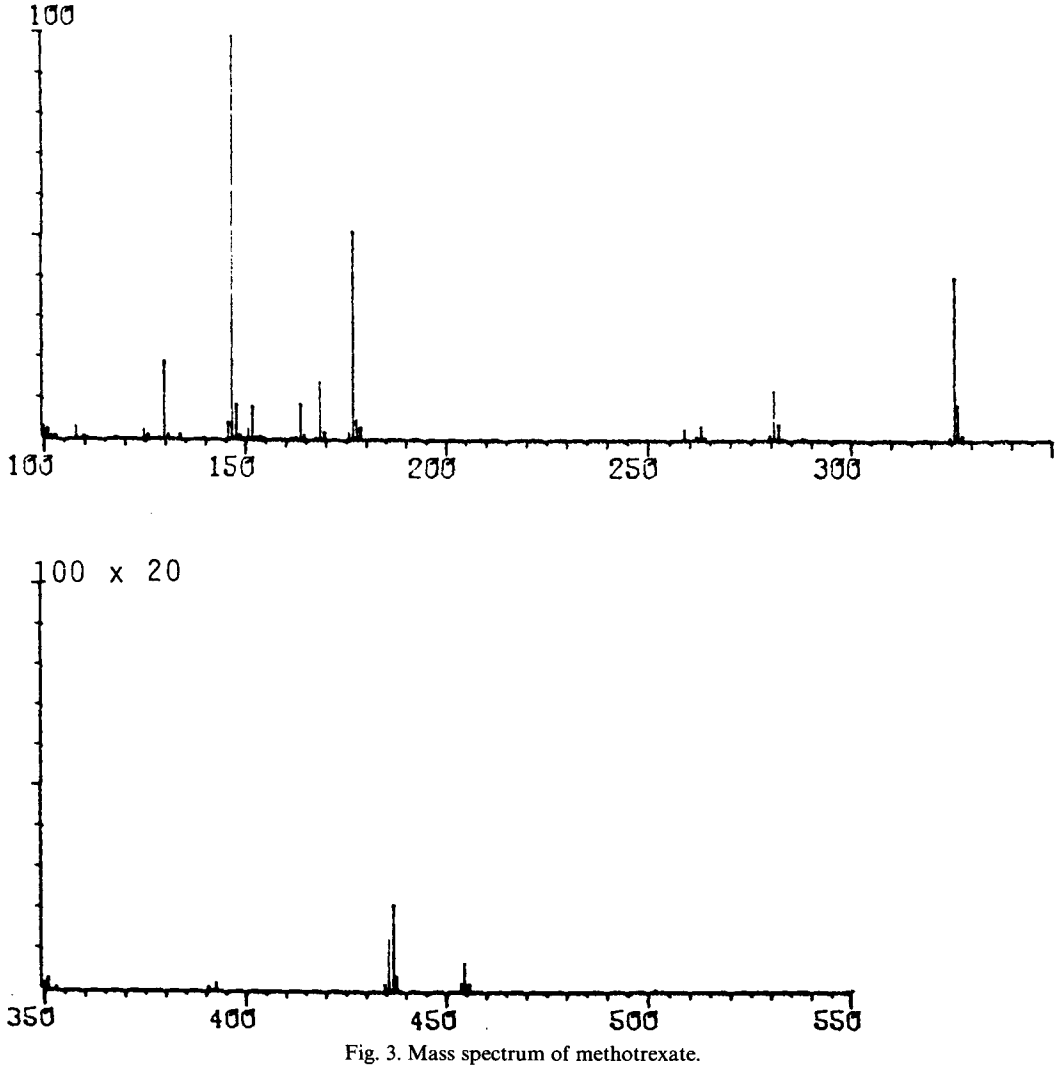

Fig. 3. Mass spectrum of methotrexate.

dence that the crystals were MTX alone, and not its complex with thymidine (Chan, 1988; Chan and Gonda, 1989).

The IR spectra of the four species show many similarities (Fig. 5); the O sample has additional bands compared to A, T and S at 3150 and 1300 cm^{-1}. Solid A has less structure at low wave numbers than the other three samples. The greatest similarity exists between the spectra of T and S. In general, lattice water absorbs at 3550–3200 cm^{-1} (antisymmetric and symmetric O-H stretching) and at 1630–1600 cm^{-1} (H-O-H bending) (Nakamoto, 1963). Since all the MTX samples are found to contain water (see below), the sharpness of the 1600 cm^{-1} band and/or the extra band at 3150 cm^{-1} for O may therefore suggest that its hydration state is different from those of other samples.

Qualitatively, the DTA and DSC traces were similar, therefore only the combined DTA-TG results are shown (Fig. 6). The traces of samples T and S are almost indistinguishable whereas both O and A have their own characteristic DTA profiles, suggesting that they are in different solid states. The gradual TG weight loss from all four samples is mostly attributable to desolvation, since the weight loss together with the initial endothermic peak (but not the subsequent peaks) disappear on cyclic runs (i.e. by heating the sample in N_2 atmosphere to the peak temperature, followed by cooling and reheating). The desolvation is confirmed in the hot-stage microscope (HSM); all samples generate bubbles in the silicone oil at elevated temperature: O, 160°C; T and S, 140–150°C, with loss of birefringence as observed by disappearance of the interference colour and extinction between cross polars accompanied by fragmentation of some crystals; A, 100°C. It should be noted that the temperature for bubble formation is higher than the DTA desolvation temperature, indicating that (i) the oil medium suppresses the desolvation and/or (ii) sufficient water droplets/

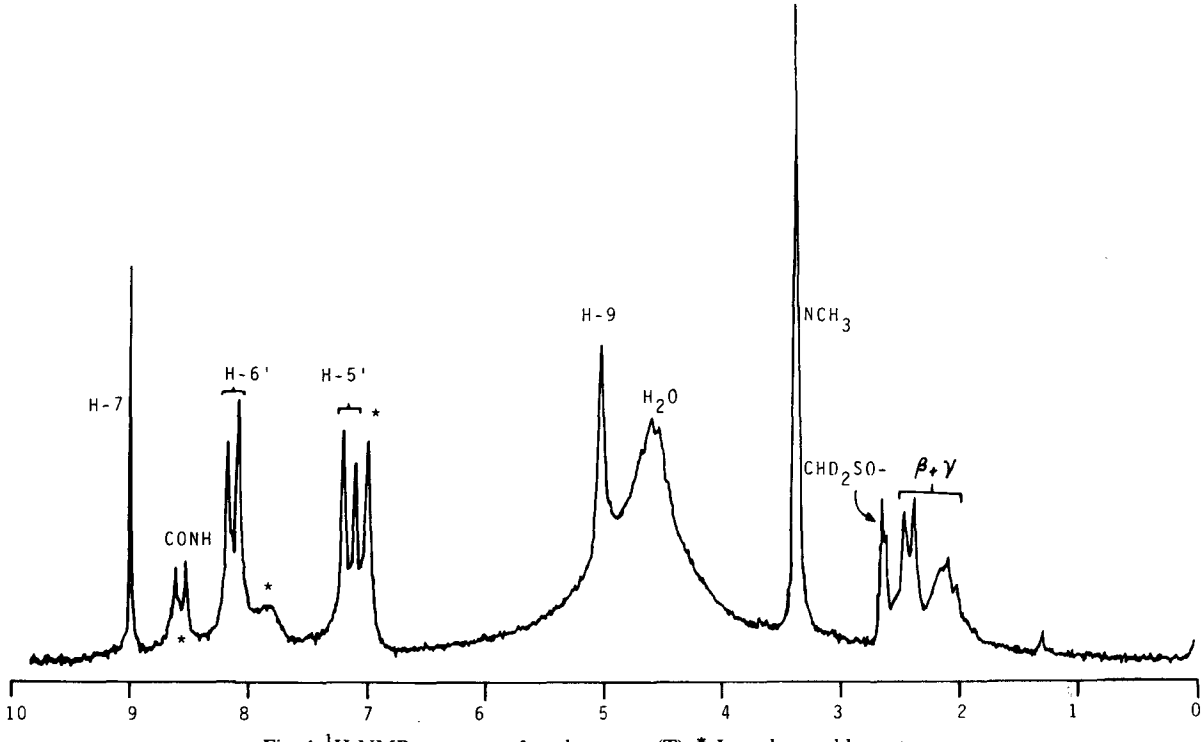

Fig. 4. ^{1}H-NMR spectrum of methotrexate (T). * Interchangeable protons.

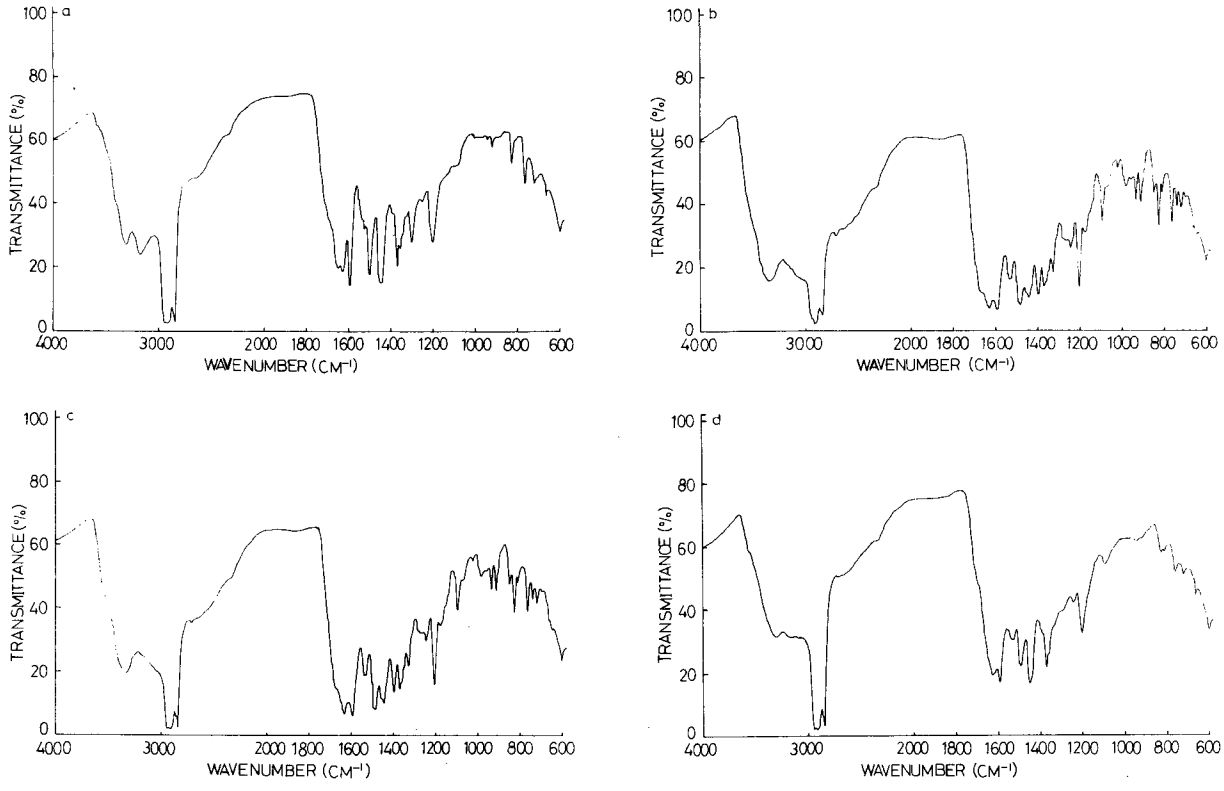

Fig. 5. Infrared spectra of methotrexate: (a) original powder (O); (b) tetragonal crystals (T); (c) spherical particles (S); (d) amorphous solid (A).

vapour is required to build up before bubbling becomes obvious at elevated temperature. Similar observations on solvates of another compound have also been reported (Stoltz et al., 1988). This hypothesis is confirmed by the absence of bubble formation on subsequent heating in silicone oil if the samples are preheated to the temperature corresponding to desolvation in DTA-TG (e.g. 120 °C for T and S; 150 °C for O).

Since A was obtained from solution of methanol, the possibility might arise that A is a methanol solvate which contributes to the desolvation reaction. This is, however, disproved by quantitative solution ^{1}H-NMR of A (by spiking the solution with a known amount of methanol) which indicates the absence of methanol at a detectability of $\leq 0.1\%$ by weight. In fact, the TG weight loss is similar to the moisture content determined by the Karl Fischer titration (Table 1) except for sample S which shows a somewhat lower value from the titration and could be ascribed to some variation in the adsorbed moisture. Unfortunately, unlike quantitative X-ray powder diffraction and quantitative spectroscopy (IR and solid-state NMR), both TG and Karl Fischer methods are unable to discriminate between adsorbed (free) and crystalline (bound) water in the samples. Nevertheless, the fact that desolvation of A occurs at a lower temperature suggests that its moisture is more loosely bound compared with other samples. Furthermore, the dehydration peak at approx. 150 °C for O suggests that its water is most tightly bound. The thermal analysis results, together with the more pronounced sharpness of the absorption in the O-H IR stretching region, suggest that the water molecules in O are more specifically and strongly located in the lattice (presumably via H-bondings). It therefore appears that O is a hydrate different from T and S (rather than a different polymorph

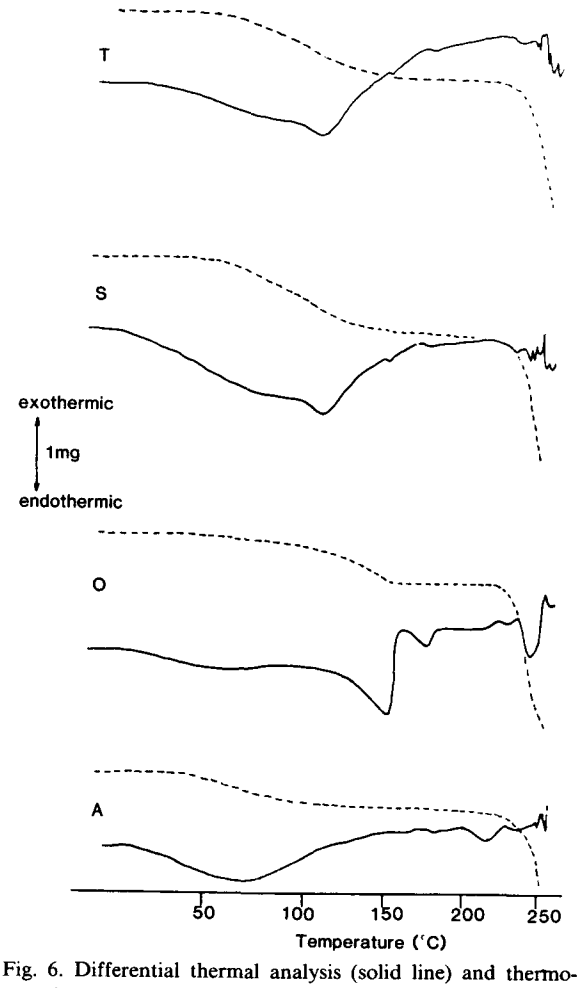

Fig. 6. Differential thermal analysis (solid line) and thermogravimetry (broken line) of four solid forms of methotrexate.

+ free water which should dehydrate more readily). The water content of T (9%) indicates that the MTX crystals in this study are likely to be dihydrate (7.3 wt% H_2O) and/or trihydrate (10.6 wt% H_2O) which is in contrast to that reported as tetrahydrate (Sutton et al., 1986). This finding suggests slight differences in solvation as a result of a different crystallization environment. In reality, due to the disorder of the glutamate side chain in the molecule which imparts disorder to the solvent molecules (as revealed by the high thermal parameters in the crystal structure), the number of water molecule per unit cell could possibly range from two to four (Hambley et al., 1986; Hambley, personal communication).

TABLE 1

Moisture content as determined by the Karl Fischer titration (weight loss by TG included in parentheses for comparison)

Sample	Moisture content (wt%)
A	5.0 (5.5)
O	6.5 (6.7)
S	7.7 (9.2)
T	8.3 (9.0)

DTA also showed a small endothermic change at approx. 175 °C which is reversible in the cyclic runs but with the peak size further diminished. Since desolvation occurs at an earlier temperature and HSM does not show any phase transition at this temperature, the most likely event would be the onset of degradation which for solid drugs usually occurs in the surface film phase at a temperature below the melting point (Guillory and Higuchi, 1962). This is further supported by a change in colour (from yellow to light brown) of the samples in the DTA pan. Furthermore, for sample T and S, a small yet reproducible kink appears at approx. 155 °C immediately following the dehydration peak. This kink is irreversible on cyclic runs and therefore could be ascribed to the collapse of the dehydrated crystal lattice involving a solid-solid transition of the crystalline to amorphous form. This hypothesis is supported by the observations that when T is pre-heated to only 120 °C (desolvation temperature) on the hot stage, the birefringence and X-ray diffraction pattern are preserved. However, on further reheating to 150 °C, the diffraction peaks disappear with loss of birefringence, i.e. characteristic of an amorphous form. The thermal changes that take place at temperature $\geq$ 200 °C are attributed to melting accompanied by decomposition with rapid weight loss resulting from charring. These are also demonstrated by HSM which shows liquidification and darkening of the samples.

Sample S gives almost identical X-ray powder diffraction pattern to T (Fig. 2). In fact, 'spherical' MTX crystals prepared by other methods (Chan, 1988; Chan and Gonda, 1989) all give the same diffraction pattern. In contrast, sample A (Fig. 2) shows a pattern characteristic of an

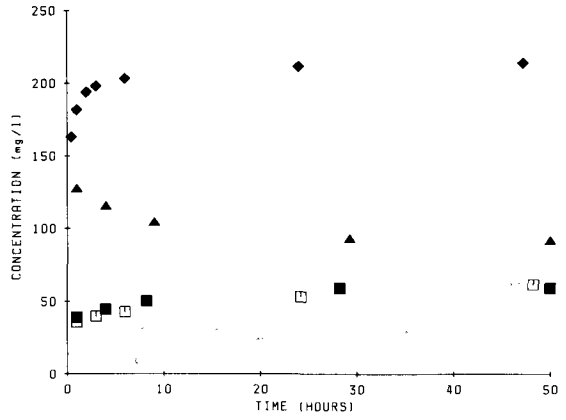

Fig. 7. Dissolution of methotrexate: original sample (O) (▲); tetragonal crystals (■); spherical particles (□); amorphous solid (◆).

amorphous solid. The original sample O (and other similar powders as, e.g., those prepared from aqueous solutions of ornithine – see above) shows a pattern (Fig. 2) suggesting the presence of crystalline matter of a different nature to that found in S and T. The pattern for O is generally broader but the sharp and split peaks at 2θ 10.7 and 17.7–18.2°, respectively, are characteristic of this sample. The results of dissolution measurements are shown in Fig. 7. As with the other physical measurements (IR spectra, X-ray diffraction and thermal analysis) samples T and S show very similar characteristics. They would therefore appear to be different crystal habits of the same pseudopolymorph (Haleblian, 1975) described as the tetragonal crystals of MTX (Hambley et al., 1986). They are highly crystalline and their relatively low solubility suggests that they are the form with the lowest energy. This is consistent with the ease with which they could be prepared, since the first crop of these tetragonal crystals was made (Chan et al., 1986). Sample A shows about 4-times higher solubility compared to T and S which agrees with its proposed amorphous nature. The persistence of high solubility of A indicates absence of any crystals in the sample which could act as seeds in a dissolution-crystallization equilibrium. The original powder (O) gives concentrations of dissolved MTX between the amorphous and tetragonal crystals, consistent with a moderate degree of crystallinity deduced from the X-ray diffraction. Also, these data are consistent with the conventional view (e.g. Attwood and Florence, 1988) that the lower the content of water of crystallization, the higher the solubility. The concentration of dissolved O gradually decreases with time suggesting a solvent-mediated transformation to the more stable hydrate T (the solubility limit of O was probably not reached). This is confirmed by the X-ray diffraction pattern of O after stirring for about 100 h in water (Fig. 2). The pattern shows superposition of the initial diffraction patterns of O and that of T. In contrast, the X-ray diffraction patterns of T, S and A remained unchanged after a similar treatment.

Conclusions

We provide evidence that MTX can exist as at least four distinct types of solid material. The original commercially available powder (O) is a solid of a low degree of crystallinity. Well formed tetragonal (T) crystals of MTX were originally obtained from aqueous solutions in the presence of thymidine (Chan and Gonda, 1989) but they can be now obtained also by slow cooling of hot saturated aqueous systems. Such crystals and their twins can be also prepared in the presence of low concentration of a number of other additives but as the concentration of these additives (e.g. β-cyclodextrin) is increased spherical (S) crystals of MTX are formed (Chan and Gonda, 1989). Physical tests show that the S solids are most likely spherical agglomerates of the T crystals. Amorphous (A) MTX has the highest aqueous solubility probably followed by O. The latter, however, can revert to T during prolonged exposure to water. This may be due to the presence of some microscopic seeds of T in the sample of O, or recrystallization in water of a different crystal form of MTX to the same hydrate as T and S.

In summary, MTX was found to exist as:

(1) a stable pseudopolymorph (hydrate) of tetragonal crystals existing as either well formed crystals (T), or as spherical particles which are agglomerates of such crystals (S).

(2) a metastable pseudopolymorph O showing a lower degree of crystallinity than T or S and the presence of crystalline matter different from T and S. This pseudopolymorph can change gradually in the presence of bulk water to T.
(3) an amorphous form of MTX.

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